Food plants and chemical ecology of sympatric species of endemic New Zealand alpine grasshoppers

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Abstract

In some habitats, multiple related species coexist without competitive exclusion. This is possibly because sympatric species have adapted to use different resources in their habitat, such as shelter and food. Sympatric species may also have sophisticated mate recognition to avoid hybridization and maximises reproductive fitness. Therefore, exploring resources, feeding and sensory structures and sexual communication systems would allow us to understand how species coexist and maintain diversity.

In central South Island, three endemic species of New Zealand alpine grasshoppers *Brachaspis nivalis*, *Sigaus australis* and *Paprides nitidus* (Orthoptera: Acrididae) occur in sympatry at high elevation (>1200 m above sea level). Past studies showed that these species are closely related and each have a preference for a particular vegetation type (rock/scree or vegetated), but their communication systems have never been explored. Insects rely on chemical cues to locate and recognize their food and mates but the majority of chemical communication systems in acridid grasshoppers are focused on economically important species (locusts). Thus, this is the first study to explore chemoreception in the New Zealand endemic grasshopper radiation.

In this thesis, I first reviewed current knowledge of chemical ecology and olfaction in acridid grasshoppers to understand the tools and techniques used to investigate chemoreception and chemical ecology in insects (Chapter 2). Then, I investigated mechanisms of the coexistence of *B. nivalis*, *S. australis* and *P.nitidus* by examining their diet, mandible morphology (Chapter 3), antennal sensory organs i.e., sensilla (Chapter 4), olfactory perception of plant volatiles (Chapter 5) and chemical profiles (Chapter 6).

The three grasshopper species were found to have broadly similar diets, sensory organs and olfactory responses to plants. Microhistological epidermal and DNA analyses (Chapter 3) showed the three species had similar food plants in their gut, with shrubs and herbs detected more often than grasses. Despite significant differences in mandible morphology, males and females eat similar plant species. Females of all species had larger mandibles than males, suggesting that they may be adapted to eating thicker plant tissues than males, and *S. australis* mandibles of both sexes were more heavily melanized than the other species indicating adaption to eating tougher plant tissues.

Five morphological types of sensilla (one taste and four olfactory receptors) were observed on the grasshopper antennae, but none were specific to a particular species or sex (Chapter 4). *Brachaspis nivalis*, however, had the fewest taste sensilla but the most olfactory sensilla compared to the other two species and showed olfactory responses to plant-derived smells even at the lowest concentration. This may be related to their rock/scree habitat where the food resources are scattered and thus require higher olfactory sensitivity (long-distance cues) than taste reception (short-distance cues) compared to more vegetated habitats of *S. australis* and *P. nitidus*. Olfactory response recordings to plant volatiles (Chapter 5) showed all three species responded more strongly to green leaf volatiles than to terpenoids, which could indicate sensitivity to plant damage rather than to plant-specific smells.

A higher abundance of olfactory sensilla was observed in male *S. australis* compared to conspecific females but no sex differences were observed in *B. nivalis* or *P. nitidus* (Chapter 4). However, female-specific compounds (oleamide and octadecanamide) were detected in cuticular hydrocarbons (CHCs) of all three species and some compounds were more abundant in particular species than in others (Chapter 6). This shows CHC composition has potential information for

mate recognition in these grasshoppers. Altogether, males and females of three New Zealand alpine grasshopper species showed similarity in their food plants, sensory organs and sensitivity to plant smells, but sexual and species differences in mandible morphology and CHC composition allow one to infer specific adaptation to food plants and sophisticated mate recognition system that explain how these three alpine grasshoppers can co-occur.

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differences between males and females within a species. Differences across species revealed from a linear model followed by a pair-wise post hoc Tukey honest significant test (Supplementary Table 4.2). Abbreviations: BF, *Brachaspis nivalis* female; BM, *B. nivalis* male; PF, *Paprides nitidus* female; PM, *P. nitidus* male; SF, *Sigaus australis* female; SM, *S. australis* male.

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Declaration

This thesis is based on publications, and the formatting style of each chapter follows the guidelines for the journal to which it will be submitted or in which it has been published or accepted for publication. Hence, there are inconsistencies in writing style and some repetition of methods and contents between the chapters presented in this thesis.

Chapter 1

General Introduction

1.1 New Zealand alpine grasshoppers

Short-horned grasshoppers belong to the insect subfamily of Caelifera (Acrididae) of orthopteran insects. They are a diverse group with over 6,700 species in 26 subfamilies and a global distribution (Song et al. 2018, 2020). Most grasshoppers are herbivorous and are important components in grassland food webs, influencing plant biodiversity and productivity (Tilman et al. 1998; Belovsky and Slade 2000, 2018). In New Zealand, most grasslands are at high elevation and most endemic grasshoppers are alpine adapted (Bigelow 1967).

There are 16 endemic New Zealand grasshopper species, 14 of which belong within four endemic genera: *Sigaus*, *Paprides*, *Brachaspis* and *Alpinacris* (Trewick and Morris 2008). They are all found in South Island, except for *Sigaus piliferus* which is found only in North Island (Bigelow 1967). Morphological traits including body colour patterns, total body length, shapes and size of the head, pronotum, antennae, male genitalia, female subgenital plate, femora and tegmina are used to classify New Zealand grasshoppers (Bigelow 1967; White 1994; Trewick 2001; Morris 2002; Trewick and Morris 2008; Dowle et al. 2014; Carmelet-Rescan et al. 2021). Phylogenetic analysis showed New Zealand alpine grasshoppers form a monophyletic clade in relation to global Acrididae species. The New Zealand radiation was dated to approximately 15 million years ago and is sister to cold-adapted species in Tasmania (Koot et al. 2020). The 14 endemic New Zealand species do not form monophyletic clades that correspond to the four described genera (Koot 2018; Koot et al. 2020; Trewick et al. 2023), and their evolutionary relationships suggest that reclassification of the New Zealand grasshopper taxa is required (Trewick et al. 2023). Better understandings of their ecology (e.g., diet, communication systems) may contribute to their systematics and conservation.

Grasshoppers in New Zealand are flightless and show colour patterns that match their background (Figure 1.1) considered to be an adaptation against visual predators (e.g., birds, lizards: Schori et al. 2019; Trewick and Morris 2008; White 1994; White and Sedcole 1991). Alpine grasshopper species predominantly occur above the tree line (>1200m above sea level: m.a.s.l.) and show physiological and behavioural adaptations to cope with harsh environments in the alpine zone (Bigelow 1967; Hawes 2015). Variation in food plants and habitat (elevation and vegetation types) preferences have been observed among these species (Table 1.1).

Figure 1.1 Grasshopper coloration of three alpine New Zealand species helps with camouflage *Brachaspis nivalis* male (**A**), *Paprides nitidus* male (**B**) and *Sigaus australis* female (**C**).

Table 1.1 Summary of habitat (elevation and vegetation) and preferred host plant species in 12 species of the New Zealand alpine grasshoppers. References: a) Bigelow, 1967; b) Koot 2018; c) Watson 1970; d) White 1994

Species	Elevation (m.a.s.l.)	Substrate	Preferred plants	References
Brachaspis nivalis	600–2000	Scree/rock	*Poa colensoi* *Veronica* spp. *Coprosma* spp.	a, b, c
Brachaspis collinus	1000–2000	Scree/rock	?	a,b
Brachaspis robustus	500–600	Scree/rock	*Elymus rectisetus* *Poa pratensis* *Achillea millefolium*	a,b
Paprides nitidus	600–1830	Mixed herb and rock	*Celmisia lyallii* *Poa colensoi* *Gaultheria depressa*	a, b, c
Paprides dugdali	400–1160	Grassland	?	a,b
Sigaus australis	1000–1800	Mixed grasses and herb/rock	*Anisotome aromatica* *Poa colensoi* *Celmisia viscosa*	a, b, c
Sigaus minutus	Lowland	?	?	a,b
Sigaus campestris	0–1550	Grassland	?	a,b
Sigaus villosus	1370–2130	Scree/rock	?	a,b
Sigaus childi	160–420	Grass, bare soil	?	a,b
Alpinacris crassicauda	1700	Herbfield	?	a,b

1.2 Ecological partitioning and grazing pressure

Ecological theory that suggests closely related species are less likely to coexist if they are using the same resources (Hardin 1960). Despite this, multiple related species of grasshoppers coexist with a high niche overlap (Joern 1979a; Joern and Lawlor 1981). Complex interactions between plant species, the herbivores, their food preferences, habitat diversity and consumer–resource cycles may enable resource-partitioning or non-equilibrium co-existence of sympatric species (Abrams and Holt 2002; Ibanez et al. 2013b). The endemic New Zealand grasshopper species *Brachaspis nivalis*, *Sigaus australis* and *Paprides nitidus* (Figure 1.2) co-occur in the mountains of central South Island (e.g., Mt. Hutt, Fox Peak, Craigieburn, Mt. Olympus) (Watson 1970; White 1975a; Koot 2018). There is some evidence of partitioning in space with *Brachaspis nivalis* more prevalent in rock/scree habitats whereas the latter two species predominate in vegetated habitats (herbfield, tussock field, or mix) (Bigelow 1967; Koot 2018). However, these three species co-occur especially in the habitats that contain both rock /scree and vegetated area (Figure 1.2A & C). All three species are primarily dicot feeders but also feed on native monocot species with soft tissues such as *Poa colensoi* and *Luzula rufa* and more than 10% of their diet can comprise flowers (Watson 1970). Past studies also suggest grasshopper feeding may be causing cumulative damage on slow-growing alpine plant species like *Celmisia sessiliflora* and *Celmisia viscosa* (White 1974a, 1975a, b, 1978) that do not flower every year (Campbell 1981). By selectively feeding on slow-growing dicots and eating flowers and seeds (plant reproductive structures) these grasshoppers could potentially have a significant impact on the composition of the native plant community. Therefore, knowing what grasshoppers eat would contribute to the

understanding of resource partitioning in sympatric species, as well as their influence on native plant community structure.

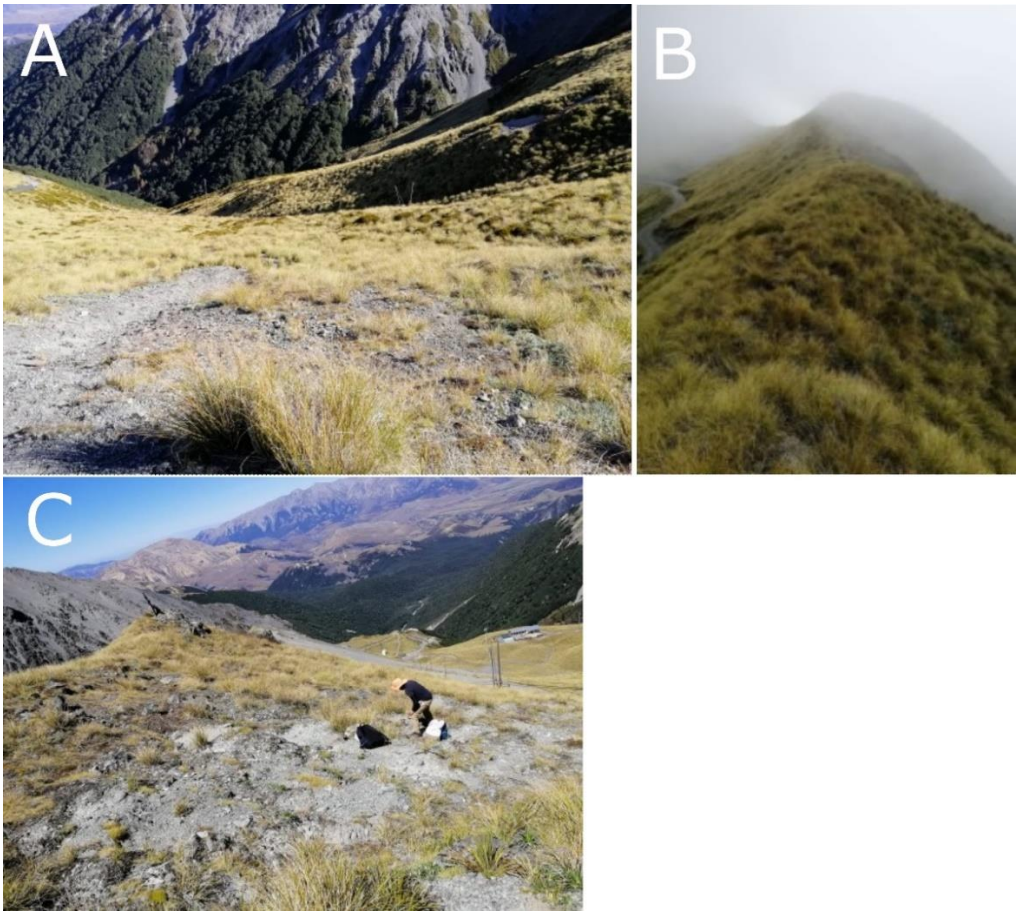

Figure 1.2 A Mixture of scree, herb and tussock field (**A, C**) and grassland (**B**) in Broken River Ski Field, Craigieburn Range.

New Zealand's native alpine grasshoppers do not communicate with sound, but conspicuous antennal movements have been observed in male grasshoppers when pursuing females (Watson 1970; Trewick personal observation). As antennae are primarily used as receivers of chemical signals (as seen in other grasshoppers: Blust and Hopkins 1987a; Chen et al. 2004; Kang and Hopkins 2004), it is probable that these grasshoppers use chemical signals to locate

and discriminate their mates and food plants. As multiple alpine grasshopper species occur on New Zealand mountains (up to five species: Koot 2018), sophisticated mechanisms are likely to be required for successful mate recognition. Insects perceive chemical signals through sensory organs called sensilla (Nakano et al. 2022), and observation of the types, abundance and distribution of sensilla allows inference of the chemoreception mechanisms in insects. Exploring sensory organs and chemical components of food plants and grasshoppers and recording olfactory responses may reveal details of the communication systems in these grasshoppers.

1.3 Thesis Outline

New Zealand's insect biodiversity has high species endemism and many studies have explored population genetic structure and species diversity, but there have been relatively few studies of the chemical ecology of New Zealand native insects (Fea et al. 2013; Nakano et al. 2019). The main reason for the lack of work is limited access to the sophisticated equipment required, such as scanning electron microscopes (SEM), gas-chromatograph coupled with mass spectrometry (GC-MS), gas-chromatograph coupled with electro-antennographic device (GC-EAD) and electroantennography (EAG). It was only possible for me to do this work by collaborating with experts at Plant and Food Research (Lincoln). My project focuses on the chemical ecology of three New Zealand alpine grasshoppers *Brachaspis nivalis, Sigaus australis* and *Paprides nitidus* and involved sensory and chemical elements to explore food plants and mate recognition among them. Although it is known that these alpine grasshoppers are somewhat segregated in terms of habitats (Table 1.1), the one study that explored their gut contents is more than 50 years old and never published beyond a thesis (Watson 1970) and communication mechanisms have never been explored. I investigated food plants in

males and females of the three species and I propose mechanisms of sensory adaptation to diet and explore their sexual communication systems.

Chapter 2: Chemical ecology and olfaction in short-horned grasshoppers were reviewed to understand chemical communication systems in grasshoppers and to identify current knowledge gaps. This chapter provides the background necessary to understand the role of chemical communication in finding food and mates in acridid grasshoppers. As New Zealand grasshoppers are silent, chemical communication is likely to be very important in this lineage. This manuscript has been published in the *Journal of Chemical Ecology*.

Chapter 3: Food plants in sympatric alpine grasshoppers were investigated by processing DNA metabarcoding with chloroplast primers (*rbc*L and *trn*L) and microhistological epidermal analysis using gut contents. Mandible morphology (size and the proportion of melanized area) was compared among species and between sexes to infer adaptation to their food plants. Identification of resource-partitioning requires detailed knowledge of food plants consumed so the objective of this chapter was to identify the species of plant eaten by the three grasshopper species when collected from the same habitat. This would allow me to determine if species- and sex-specific use of resources involved differential food plant choice by adults.

Chapter 4: Types, distribution and abundance of sensilla on antennae were observed using a scanning electron microscopy (SEM) to explore different sensory capabilities among species and between sexes of alpine grasshoppers. Species that

rely on chemical communication for feeding and mating could be specialised for the detection of short- or long-range chemical cues and species- and sex-specific differences of antennal sensilla offer opportunity to identify niche differentiation and sex roles. The objective of this chapter was to provide a basic understanding of sensory systems in the three grasshopper species to aid studies of food (Chapter 5) and mate (Chapter 6) recognition systems. This manuscript has been published in *Zoomophology.*

Chapter 5: Sensory adaptation to food plants was explored by analysing chemical components of grasshopper food plants and recording electrophysiological responses to plant chemicals. Sympatric species are expected to have species-specific preferences for particular plants to avoid competition (Chapter 3) and the ability to recognise their favourite food. The objective of this chapter was to identify species-specific sensitivity to plant volatiles that may correspond to their antennal sensilla abundance (Chapter 4), plant preferences, and habitat vegetation composition.

Chapter 6: S**exual communication systems** were explored by analysing cuticular hydrocarbons (CHCs) to identify species and/or sex specific chemicals that might be used for sexual communication. Characterising species- and sex-specific chemical profiles allowed me to understand whether or not these species rely on chemical cues in mate recognition, their sex roles, and whether long-distance (volatiles) or short-distance (CHCs) signals are involved. This would contribute to the understanding of mechanisms that facilitate maintenance of species diversity within a habitat.

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In which chapter is the manuscript/published work?	2
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Chapter 2

Chemical ecology and olfaction in short-horned grasshoppers (Orthoptera: Acrididae)

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ORIGINAL RESEARCH - RESUBMISSION

Chemical Ecology and Olfaction in Short-Horned Grasshoppers (Orthoptera: Acrididae)

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Abstract

Chemoreception plays a crucial role in the reproduction and survival of insects, which often rely on their sense of smell and taste to find partners, suitable habitats, and food sources, and to avoid predators and noxious substances. There is a substantial body of work investigating the chemoreception and chemical ecology of Diptera (flies) and Lepidoptera (moths and butterflies); but less is known about the Orthoptera (grasshoppers, locusts, crickets, and wēta). Within the Orthoptera, the family Acrididae contains about 6700 species of short-horned grasshoppers. Grasshoppers are fascinating organisms to study due to their significant taxonomic and ecological divergence, however, most chemoreception and chemical ecology studies have focused on locusts because they are agricultural pests (e.g., *Schistocerca gregaria* and *Locusta migratoria*). Here we review studies of chemosensory systems and chemical ecology of all short-horned grasshoppers. Applications of genome editing tools and entomopathogenic microorganism to control locusts in association with their chemical ecology are also discussed. Finally, we identify gaps in the current knowledge and suggest topics of interest for future studies.

Keywords Chemoreception · Chemical ecology · Acrididae · Sensilla · Volatiles · Chemical-mediated behaviors

Introduction

The insect order Orthoptera comprises crickets, katydids, wētā, and grasshoppers – a group that exhibits a great diversity of communication methods. Acoustic communication is well-developed in the suborder Ensifera (crickets and katydids) with about 15,500 described species using acoustic signals (Song et al. 2020). Although the majority of Caelifera (grasshoppers, locusts, and their allies) can hear, acoustic communication is less common in this group and generally less sophisticated (Song et al. 2020). However, the Caelifera do use an array of complex chemical signals for communication and foraging. Within the suborder Caelifera, the short-horned grasshoppers (Acrididae) comprise more than 6700 species described worldwide (Song et al. 2018), found in a wide range of habitats (boreal to sub-alpine zones: Ibanez et al. 2013a, 2013b; Joern 1979; Koot et al. 2020; Sergeev 2011), and displaying a wide range of diets (e.g., monophagous vs. polyphagous; forbivorous vs. graminivorous: Isely 1944; Joern 1979), and sexual communication systems (acoustic, visual and chemical: Finck et al. 2016a, 2016b; Hassanali et al. 2005; Song et al. 2020). This diversity provides an excellent opportunity to review what we know of the chemical ecology and chemoreception of short-horned grasshoppers.

To date the majority of research on grasshopper habits has been directed towards a few locust species because of their economic importance (e.g., the desert and migratory locust: *Schistocerca gregaria* and *Locusta migratoria*) despite the ecological and taxonomic diversity of the group. Locusts are notorious agricultural pests that display phase polyphenism (Pener and Simpson 2009). When resources (mates, food plants, perches, oviposition sites) are widely dispersed individual locusts are also dispersed (the solitarious phase), but locusts shift to a gregarious phase when resources are clumped (Fig. 1). Outbreaks of the gregarious phase cause considerable agricultural loss as swarms of locusts damage crops. The switch between the two phases is mediated by chemical signaling with environmental and conspecific cues stimulating rapid shifts (within a few hours) in gene expression, biochemistry, and behavior, and more gradual changes (lifetime or trans-generation) of morphology and physiology (Fig. 1). Trying to understand the cues that result in switches

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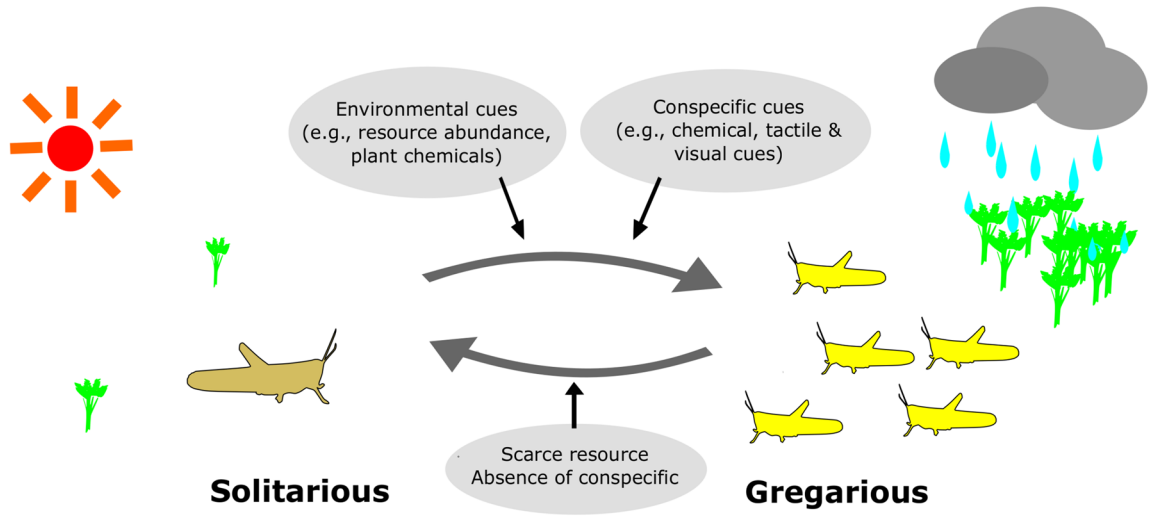


	Solitarious	Gregarious
Gene expression	Expression of genes involved in repulsive behavior [a,b,e,f]	Expression of genes involved biosynthesis & perceptions of gregarious pheromones [a-d,g]
Hindgut microbial community	*Enterococcus* [h]	*Enterobacter* [h]
Behaviors	Avoid conspecifics [a,b,e,f,i] Sedentary [i,j]	Attracted towards conspecifics [a,b,e,f,i] Highly migratory [i,j]
Communication sytem	Cryptic [k-m] Long-range signals (smells [n,o] and vision [o])	Release gregarious pheromones [k-m] Short-range smells [n,o], touch [i] and taste?
Morphology	Higher sensilla abundance [p,q] Cryptic color, larger eyes and body [r,s]	Lower sensilla abundance [p,q] Bright color, smaller eyes and body [r,s]

Fig. 1 The environmental and conspecific signals that contribute to locusts switching between their solitarious and gregarious phases. References: **a** Guo et al. 2011, **b** Guo et al. 2018b, **c** Wei et al. 2019, **d** Li et al. 2016, **e** Ma et al. 2015, **f** Ma et al. 2019, **g** Chen et al. 2018, **h** Lavy et al. 2019, **i** Rogers et al. 2014, **j** Maeno et al. 2016; **k** Wei et al. 2017, **l** Amwayi et al. 2012, **m** Njagi et al. 1996, **n** Inayatullah et al. 1994, **o** Ould Ely et al. 2006, **p** Ochieng et al. 1998, **q** Greenwood and Chapman 1984, **r** Sugahara et al. 2015, 2017, **s** Gordon et al. 2014; Rogers et al. 2010

in locust phase has therefore driven research into Acrididae chemical ecology. In addition, because insecticides used to control locusts have negative impacts on human health and the environment (Byers 1991; Zhang et al. 2019), the use of alternative control methods such as pheromone traps, genetically modified pests, and entomopathogenic fungi, that are more species-specific and environmentally safer are being investigated.

As chemical communication has a central role in triggering the switch between the two locust phases (Hassanali et al. 2005), the study of chemical ecology provides the basis for predicting when swarming is likely to happen and potentially controlling outbreaks. At sensilla, which are the sensory organs that project through the insect exoskeleton, sensory neurons and proteins respond to specific tastes or smells. Pioneering studies identified compounds involved in gregarization (i.e., aggregation pheromones) using gas-chromatograph and mass spectrometry (GC-MS), and explored the mechanisms of perception using physiological and behavioral observations (Mahamat et al. 1993; Hansson et al. 1996; Ochieng et al. 1998; Niassy et al. 1999; Ochieng and Hansson 1999). Physiological responses to specific chemical stimuli can be investigated using electrophysiological techniques including electroantennography (EAG) (Torto et al. 1994; Njagi et al. 1996; Chen et al. 2004) and single sensillum recordings (SSRs) (Altner et al. 1981; Ochieng and Hansson 1999; Cui et al. 2011; You et al. 2016). These techniques monitor neuron response by inserting an electrode into an antenna (EAG) or a sensillum (SSRs) while the insect is exposed to a particular chemical compound.

More recently, molecular tools including transcription and genome editing have been employed to investigate chemical signaling (Guo et al. 2014, 2020; Zhang et al. 2015, 2017; Jiang et al. 2018). In these studies, genes that are potentially involved in the perception and biosynthesis of pheromones have been identified by observing expression patterns in sensory tissues and sensilla with quantitative real-time PCR (Jin et al. 2005; Zhang et al. 2015; Chen et al. 2018; Yuan et al. 2019; Li et al. 2020) and in situ hybridization (Yang et al. 2012; Jiang et al. 2017, 2018). The functional diversity of chemoreceptive proteins has been deciphered by silencing candidate genes using RNA interference (Guo et al. 2011; Wei et al. 2019) and CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) (Li et al. 2016; Chen et al. 2018; Guo et al. 2020). Together these chemical, physiological, behavioral, and molecular approaches have extended our understanding of chemical ecology and chemoreception in grasshoppers.

The detection of food, competitors, predators, and mates involves olfaction or contact-chemoreception (gustation) by discrimination of volatile or soluble stimulants (Sánchez-Gracia et al. 2009). Our review explores what is known about these mechanisms and their roles in short-horned grasshoppers (Orthoptera; Caelifera; Acrididae) with particular reference to: 1) communication systems in acridid grasshoppers, 2) the chemical signals they perceive (pheromones, cuticular hydrocarbons, and plant-derived chemicals) and their effect on grasshoppers' behaviors, 3) types and distribution of sensilla, 4) chemoreception-associated proteins, and 5) applied chemical ecology in pest control and conservation. We identify knowledge gaps and suggest useful and rewarding avenues for future research.

Communication Systems in ACRIDID Grasshoppers

Multi-Modal Communication Systems in Grasshoppers Grasshoppers communicate using visual, auditory, tactile, and chemical signals (Perdeck 1958; Ritchie 1990; Chen et al. 2004; Hassanali et al. 2005; Finck et al. 2016b; Finck and Ronacher 2017; Song et al. 2020). Acoustic mate communication is important for many species within the subfamilies Gomphocerinae and Oedipodinae (Song et al. 2018, 2020), and some of these grasshoppers are known to also use chemical signals. For example, some *Chorthippus* grasshopper species (Gomphocerinae) use acoustic signals for long-range communication and chemical signals at short-range (Perdeck 1958; Ritchie 1990; Finck et al. 2016b; Finck and Ronacher 2017). In sympatric *C. biguttulus* and *C. mollis*, acoustic signals enable long-range recognition of conspecific males and short-range chemical signals are used to detect conspecific females (Finck et al. 2016b; Finck and Ronacher 2017). Multimodal signaling of this sort allows assortative mating and differ among even closely related lineages (Neems and Butlin 1995; Finck et al. 2016a; Finck and Ronacher 2017). For instance, *Chorthippus parallelus erythropus* females use olfaction in mate choice resulting in positive assortative mating, while females of their close relatives *C. parallelus parallelus* use male's songs to select their partners (Ritchie 1990).

Locust species use visual, tactile, acoustic, and chemical cues to locate and recognize predators, intraspecific phase, sex, and developmental stages. The use of long-range and short-range signals is phase-dependent. The gregarious phase occurs when the grasshoppers are in high density suggesting an emphasis on close range or contact signaling (Ferenz and Seidelmann 2003; Rogers et al. 2003; Hassanali et al. 2005). Touch is known to mediate behavioral phase shifts and is used as a cue for measuring rapid changes of behavior and gene expression in artificially crowded solitary and isolated gregarious locust individuals (Ould Ely et al. 2006; Guo et al. 2011; Rogers et al. 2014; Li et al. 2016). Detection of smells at short-range (up to 150 cm) in gregarious locusts has also been demonstrated (Inayatullah et al. 1994; Ould Ely et al. 2006). Conversely, for locusts at low population density (solitarious phase), long-range signals like smells and sound are likely to be more important (Inayatullah et al. 1994; Ferenz and Seidelmann 2003; Hassanali et al. 2005; Ould Ely et al. 2006). Elevated sensitivity to high frequency-sound was observed in solitarious *S. gregaria* which might allow detection of bat echolocation (Gordon et al. 2014). When the temperature is favorable solitarious locusts are active at night (Gaten et al. 2012; Gordon et al. 2014); and this might expose them to bat predation more than day-time active gregarious locusts. Solitarious locusts are repelled by conspecifics presumably to minimize competition in resource-limited conditions (Ma et al. 2015, 2019; Guo et al. 2018b), but they are attracted to each other for mating (Inayatullah et al. 1994; Ould Ely et al. 2006). Thus, long-range signals influence conspecific avoidance and mate location in solitarious locusts, demonstrating that ecological circumstances shape communication systems.

Chemical Communication in Grasshoppers Chemical signals are important for the survival and reproductive success of phytophagous insects. In grasshoppers, chemical signals facilitate sexual reproduction by providing cues for the recognition of conspecifics (Neems and Butlin 1994, 1995; Tregenza et al. 2000a, 2000b; Finck et al. 2016b), their sex (Njagi and Torto 1996, 2002; Tregenza et al. 2000a; Stahr et al. 2013; Finck et al. 2016b; Stahr and Seidelmann 2016) and their quality (Stahr et al. 2013; Stahr and Seidelmann 2016). Insect-derived chemical signals are either cuticular hydrocarbons (CHCs) or volatiles. Cuticular hydrocarbons are detected either by direct contact or over short distances.

Chorthippus grasshopper males use CHCs to identify potential mates and have been observed touching the body and antennae of females with their antennae before copulation (Ritchie 1990; Finck et al. 2016b). Conversely, olfactometer studies show that detection of odour cues results in the attraction of many grasshopper species towards volatiles of conspecifics (*L. migratoria*: Guo et al. 2011; *S. gregaria*: Inayatullah et al. 1994; Ould Ely et al. 2006; *Schistocerca americana*: Stahr et al. 2013; *Dociostaurus maroccanus*: Guerrero et al. 2019) and host plants (*S. gregaria*: Njagi and Torto 1996; *Melanoplus sanguinipes*: Hopkins and Young 1990). The specific chemical compounds identified and their behavioral effect is described in the next section.

Chemical Signals Perceived and their Effect on Grasshopper Behavior

Cuticular Hydrocarbons (CHCs) Cuticular hydrocarbons are derived from the insect exoskeleton and have a primary function of preventing water loss (Blomquist et al. 2018). Cuticular hydrocarbons are relatively long carbon chains (21 to >40 carbons) with single (alkanes) or double bonds (alkenes and alkadienes) sometimes including methyl branches (Gibbs and Rajpurohit 2010; Blomquist et al. 2018). In *L. migratoria*, straight-chain 25–33 carbon alkanes are important water-proofing agents, and disruption of CHC synthesis results in high mortality due to severe water-loss (Yu et al. 2016). Cuticular hydrocarbons also provide a barrier against fungi and insecticides (Wu et al. 2020; Zhang et al. 2021).

Short-horned grasshoppers have species-specific qualitative and quantitative CHC profiles. The locusts *S. gregaria* and *L. migratoria migratodiodes* each have characteristic CHCs (Lockey and Oraha 1990) with the former species dominated by straight-chain alkanes whereas the latter dominated by mono- and dimethyl-alkanes (with some compounds being specific). In the sympatric grasshoppers *C. biguttulus* and *C. mollis*, 34 carbon straight chain, and methyl-branched alkanes have been identified (Finck et al. 2016a), with the position of the methyl group in di- and tri- methyl branched alkanes differing between species and sexes (Perdeck 1958; Finck et al. 2016a; Finck and Ronacher 2017).

When CHCs are used in communication, they are usually considered to be short-range or contact-chemical signals, as opposed to volatiles that provide long-distance cues (Blomquist et al. 2018). Cuticular hydrocarbons generally have lower volatility and higher melting points than pheromones, resulting in CHC molecules remaining close to the insect while volatiles diffuse through the air (Menzel et al. 2017, 2019). However, it is unclear how the chain length or molecular weight of these molecules relates to use in contact-chemical communication versus olfactory reception. *Chorthippus* grasshoppers use both contact and short-range (5–10 mm) olfactory reception when perceiving 25–39 carbon straight-chain and methyl-branched alkanes (Finck et al. 2016a, 2016b), suggesting that CHCs can be perceived through olfaction to some degree. The perception distance is likely to be species-specific and depend on the number of sensilla, olfactory sensory neurons (see CHEMORECEPTION) and environmental conditions (e.g., wind speed, temperature).

Cuticular hydrocarbons used for communication are subject to sexual and natural selection. Assortative mating for similar CHC profiles is seen within *C. parallelus* that comprises several subspecies in the Pyrenees mountains (Neems and Butlin 1995; Tregenza et al. 2000a, 2000b). Population-level differences in CHCs have been detected, with eastern *C. parallelus* having a higher proportion of long-chain vs. short-chain hydrocarbons than western populations (Neems and Butlin 1995). The drier eastern environment compared to the west, may explain CHC differences as long-chain hydrocarbons may provide greater waterproofing to the grasshoppers reducing desiccation. Host plant preference can also lead to CHC divergence. The north American grasshopper *Hesperotettix viridis* feeds on either *Gutierrezia* snakeweed or *Solidago* golden-rod, and assortative mating with respect to the host plant has been detected (Grace et al. 2010). This mate choice appears to derive from grasshopper CHC profiles that correlate with food plant (Grace et al. 2010), but the grasshoppers also have different body colors depending on their host plants and therefore the use of visual cues in mate selection cannot as yet be excluded.

Volatile Pheromones Volatile pheromones contribute to the detection and choice of mates (sex pheromones) but also have other roles (e.g., primer pheromones, alarm, aggregation) (Jacobson 1972; Howse et al. 1998). While some CHCs function as short-distance pheromones (especially short-chain CHCs), volatile pheromones usually function as long-distance chemical cues (Blomquist et al. 2018). The quality and quantity of the pheromones produced can communicate the quality of potential mates including mating status (Burke et al. 2015; Tabata et al. 2017), feeding condition (Barry et al. 2010), and reproductive mode (i.e., sexual or asexual: Burke et al. 2015; Tabata et al. 2017) as well as their location.

Studies of locust pheromones have revealed volatiles involved with courtship inhibition (Seidelmann and Ferenz 2002), premating behavior (Njagi and Torto 2002), oviposition (Saini et al. 1995; Rai et al. 1997), aggregation (Dillon and Charnley 2002; Dillon et al. 2002; Guo et al. 2020), maturation acceleration (Mahamat et al. 1993; Stahr et al. 2013), and sexual selection (Njagi and Torto 1996; Seidelmann and Ferenz 2002; Stahr and Seidelmann 2016; Wei et al. 2017,

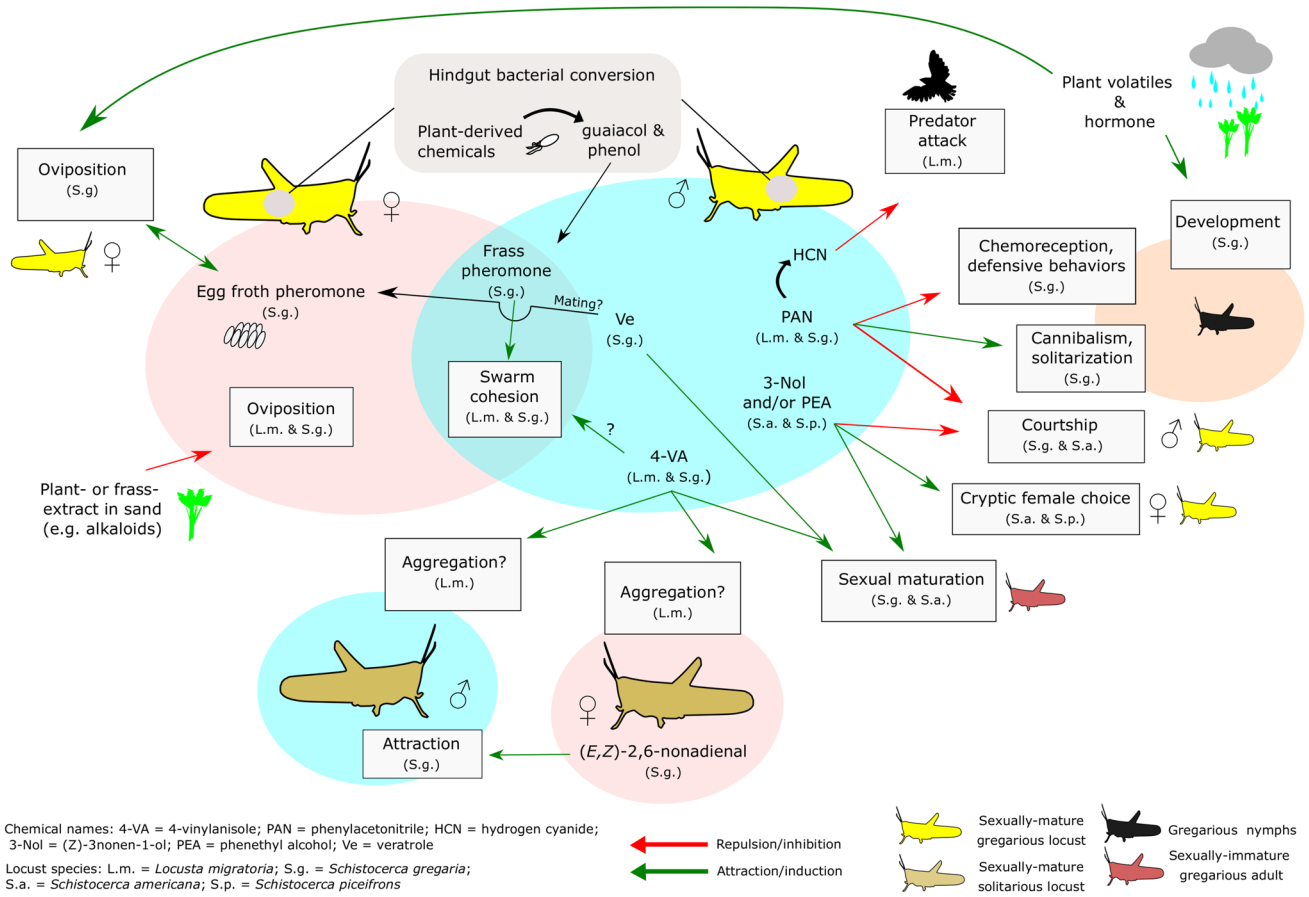


Fig. 2 Chemical ecology of phase shifts in well-studied locust species (*Locusta migratoria*, *Schistocerca gregaria*, *S. americana* and *S. piceifrons*). Compounds that are inside the blue circle are the major component of male pheromones, in pink circles are female pheromones, and where circles overlap are compounds shared by males and females

2019; Guo et al. 2020) (Fig. 2). Although several compounds such as veratrole, guaiacol, benzaldehyde, hexanoic acid, nonanal, and (*Z*)-3-nonen-1-ol are found in the pheromonal blends of different locust species (e.g., *S. americana*, *S. gregaria*, *S. piceifrons*, *L. migratoria*), significant differences in emission dynamics are observed within and among them (Niassy et al. 1999; Mahamat et al. 2000; Stahr et al. 2013; Stahr and Seidelmann 2016; Wei et al. 2017). Relative concentrations in pheromone cocktails provide signals that are specific to species, sex, and developmental stages in locusts.

Most of these studies have focused on volatiles associated with the gregarious phase with the intention of improving pest control and reducing crop damage. The only pheromone documented for the solitarious locust is (*E,Z*)-2,6-nonadienal released by solitarious female *S. gregaria*, and is known to induce electro-physiological response and behavioral attraction by males (Ochieng and Hansson 1999; Ferenz and Seidelmann 2003).

Pheromones associated with the gregarious phase have been found in several locust species (Fig. 2). 4-vinylanisole is an aromatic compound produced by gregarious *L. migratoria* and *S. gregaria* and higher emission is observed in adult males than in females or immature locusts in this phase (Mahamat et al. 2000; Wei et al. 2017). Although produced only by gregarious individuals 4-vinylanisole influences aggregation behavior as an attractant for both gregarious and solitarious *L. migratoria* (Guo et al. 2020). In *S. piceifrons*, gregarious male-specific volatiles, phenethyl alcohol, and (*Z*)-3-nonen-1-ol induce neither attraction nor repulsion (Stahr and Seidelmann 2016). However, hatching success of eggs was higher after copulation with scented (either or both phenethyl alcohol and (*Z*)-3-nonen-1-ol) solitarious males or gregarious males compared to unscented solitarious males. This shows that these compounds are used in cryptic female choice in *S. piceifrons* (Stahr and Seidelmann 2016) and (*Z*)-3-nonen-1-ol has a similar role in *S. americana* (Stahr et al. 2013). However, in *S. americana*, it is also implicated in mate-guarding and induction of sexual maturation (Stahr et al. 2013).

Locusts have unusual group-behavior during oviposition (Saini et al. 1995; Rai et al. 1997; Tanaka and Sugahara 2017; Tanaka et al. 2019), synchronous egg-hatching

(Tanaka et al. 2018; Sakamoto et al. 2019), and development (Ellis et al. 1965; Mahamat et al. 1993; Stahr et al. 2013). These group behaviors are thought to help secure resources (e.g., food, oviposition substrate) and enhance defence against predators (Hassanali et al. 2005), and at least some are controlled by pheromone signals. In *S. americana*, the same compound that is used for cryptic female choice ((*Z*)-3-nonen-1-ol) accelerates the sexual maturity of newly moulted adults (Stahr et al. 2013). Veratrole, the major chemical component of gregarious males, also induces sexual maturation (yellowing and copulation) in *S. gregaria* (Mahamat et al. 1993, 2000; Seidelmann et al. 2003). Veratrole is also found in egg froth from female *S. gregaria* and induces gregarious females to oviposit (Saini et al. 1995; Rai et al. 1997). Locusts are selective in oviposition sites as it affects hatching rate and embryonic development (Tanaka and Sugahara 2017; Tanaka et al. 2019). Laying eggs at sites used by conspecific females may increase the chance of successful embryonic development and hatching. As veratrole is absent or only in trace amounts in the pheromonal component of female *S. gregaria* (Torto et al. 1994) it may be transferred by male contact during swarming or copulation.

The compound phenylacetonitrile was initially considered an aggregation pheromone as it is released most by gregarious rather than solitarious locusts (Njagi et al. 1996; Niassy et al. 1999), however, it is now known to repel conspecifics of *L. migratoria* and *S. gregaria* (Seidelmann and Ferenz 2002; Seidelmann et al. 2005; Wei et al. 2019). Phenylacetonitrile is the dominant compound released by gregarious males of both *S. gregaria* (80%: Mahamat et al. 1993) and *L. migratoria* (>30%: Wei et al. 2017). Emission dynamics of phenylacetonitrile also differ between sexes and developmental stages within each species (Mahamat et al. 1993; Niassy et al. 1999; Wei et al. 2017). In *L. migratoria*, it appears that phenylacetonitrile can be used to deter predators as the compound is a precursor of hydrogen cyanide (toxic to vertebrates; Wei et al. 2019). Gregarious locusts may be at higher risk of predation compared to solitarious locusts as they are more colorful and occur in high density (Hassanali et al. 2005). Thus, a higher emission of phenylacetonitrile in gregarious vs. solitarious locusts is considered to be an adaptation to group-living. In *S. gregaria*, phenylacetonitrile acts as a courtship inhibitor to guard mates after copulation and to avoid homosexual attacks in a large swarm (Seidelmann and Ferenz 2002). Phenylacetonitrile has negative effects on the nymphs of *S. gregaria*, as it disrupts chemoreception, reduces immune systems and feeding rate, and induces behavioral disorientation (Bashir and Hassanali 2010; Abdellaoui et al. 2020). In a field study, groups of marching gregarious nymphs were sprayed with phenylacetonitrile solution (Bashir and Hassanali 2010). The marching groups started to lose coherence two days after the application (possibly due to disruption in olfaction), and enhanced predation rate observed associated with reduced defensive behavior. Intriguingly, cannibalistic behaviors were also observed among phenylacetonitrile-treated nymphs although the reason behind this remains unclear.

Plant-Derived Signals Phytophagous insects adapt to the chemical components of host plants in terms of recognition (e.g., green leaf volatiles) and response to plant defence (alkaloids, flavonoids) as well as nutrient composition (crude proteins, starch, and lipids). Many secondary metabolites of host plants are toxic to phytophagous insects, inhibiting their development and reproduction, and detoxification can be energy expensive and involve specialized biochemical pathways that lead to dietary specialization in insects (Ibanez et al. 2013a; Huang et al. 2017, 2020; Giron et al. 2018; Cui et al. 2019a). Short-horned grasshoppers display a wide range of feeding patterns with some species being absolute specialists (e.g., creosote bush grasshopper *Bootettix argentatus*: Chapman et al. 1988) while others are oligophagous or polyphagous (locust species). Different grasshopper species, therefore, display a range of responses to plant toxins involving recognition and avoidance, detoxification, or food mixing (polyphagy) to mitigate quantitative effects (Giron et al. 2018; Cui et al. 2019a).

The toxic, repellent, or feeding deterrent effects of plant synthesised compounds on grasshoppers have been tested in numerous studies. For example, the addition of certain flavonoids that are common secondary metabolites in plants (needlegrass *Stipa krylovii* and false wheatgrass *Leymus chinensis*) favored by the grasshopper *Oedaleus asiaticus* reduces its growth and survival (Cui et al. 2019a). Elevated transcription of enzymes involved in detoxification has been observed when *O. asiaticus* feed on plants with high levels of phytotoxins including flavonoids, terpenoids, alkaloids, and tannins (Huang et al. 2017, 2020). The creosote bush *Larrea tridentate* is equipped with anti-digestive resin of which a major component is a phenolic aglycone called nordihydroguaiaretic acid (NDGA). When NDGA is applied to the leaves of Jojoba *Simmondsia* under experimental conditions it increases the acceptability of this plant to the creosote-grasshopper *B. argentatus* that does not normally feed on it but deters the grasshoppers for which *Simmondsia* leaves are a preferred food (*Ligurotettix conquilletti* and *Clibolacris parviceps*; Chapman et al. 1988). Thus, although NDGA is probably a plant defense it can also be considered a plant-derived signal for grasshoppers discriminating host and non-host plants.

Similarly, plant-derived chemicals can act as signals for grasshopper biology such as molting and reproduction. During the dry season when the plants are senescent, the production of the plant hormone, gibberellic acid (a plant growth regulator) decreases. The shortage of this chemical delays ecdysis and egg-laying in *S. gregaria* two-fold

or more (Ellis et al. 1965; Carlisle et al. 1969). When gibberellic acid is added to the senescent leaves, it accelerates sexual maturation in *S. gregaria* but interestingly, delayed development was observed when the compound was added to green leaves (Ellis et al. 1965). The delayed development is possibly related to the phytotoxicity of gibberellic acid above a certain threshold. For example in *L. migratoria*, the rate of consumption, nymphal development, and oviposition of newly emerged females reduced as the concentration of this compound increased (Abdellaoui et al. 2009, 2015). These studies show that gibberellic acid signals optimal time for development and reproduction, but it also can be toxic above certain limits.

Plant-derived chemicals can influence where grasshoppers oviposit their eggs. *Schistocerca gregaria* females presented with a choice of sand containing either leaf extracts of their host plants (orchard grass, cabbage, sorghum, romaine lettuce, Japanese mustard spinach, or silver grass), frass extract from other locusts (*S. gregaria*, *L. migratoria,* and *Patanga succincta*) or water (control), laid more eggs in the control sand than in the sand containing extracts (Tanaka et al. 2019). This preference for oviposition sites is related to egg-hatching rate and embryonic development (egg size and antennal length) that were significantly reduced by the presence of frass and plant extracts. A similar inhibition effect has been observed in *L. migratoria* (Sugahara et al. 2021), the choice of oviposition sites was not influenced by phase polyphenism or bacterial activity in either species (Tanaka et al. 2019; Sugahara et al. 2021). The compounds inducing oviposition inhibition are unknown but may involve toxic compounds such as alkaloids.

Chemical Biosynthesis in Grasshoppers In insects, CHCs are synthesised from fatty acids and terpenoid lipids, in specialized cells called oenocytes present in the abdomen or fat bodies (Blomquist et al. 2018). Synthesis of different types of CHCs involves a variety of catalysts including fatty acid synthase, reductases, and elongases (Blomquist et al. 2018). In short-horned grasshoppers, biosynthetic pathways of CHC formation have only been studied in *L. migratoria* (Yu et al. 2016; Wu et al. 2020) and *S. gregaria* (Diehl 1975). Two genes from the superfamily of cytochrome P450 enzymes are expressed specifically in oenocytes and they are responsible for catalyzing the synthesis of 25–33 carbon alkanes and mono- and di-methyl branched alkanes (Yu et al. 2016; Wu et al. 2020).

In insects such as Coleoptera, Lepidoptera, and Diptera, pheromones are synthesized in a pheromone gland commonly located in the abdomen (Blomquist et al. 2018), but the production of pheromones in locusts involves specialized epidermal cells in several different body parts (abdomen, legs and/or wings; Seidelmann et al. 2003; Amwayi et al. 2012; Fürstenau et al. 2013; Stahr and Seidelmann 2016). This has been demonstrated by injecting radio-labelled precursor compounds into the tissue of dazed specimens (Fürstenau et al. 2013) or isolated tissues (Seidelmann et al. 2003). As with CHCs, its synthesis involves specialized enzymes (Blomquist et al. 2018). An antipredator pheromone in *L. migratoria*, phenylacetonitirile, and its derivative (hydrogen cyanide) are synthesized with an enzyme from the same gene family as enzymes involved in CHC synthesis (cytochrome P450) (Wei et al. 2019). Thus, cytochrome P450 enzymes known for their roles in the metabolism of toxic compounds (including insecticides) are also important in the production of both CHCs and pheromones. A further pathway in pheromone synthesis involves Enterobacteriaceae gut bacteria. Even when isolated and applied to sterile frass, *Pantoea* and *Klebsiella* bacteria generated guaiacol and phenol that are known to be cohesion pheromones in gregarious *S. gregaria* (Obeng-Ofori et al. 1994; Dillon et al. 2002). These compounds are also observed in other locust species (*S. americana*: Stahr et al. 2013; *S. piceifrons*: Stahr and Seidelmann 2016; *L. migratoria*: Shi et al. 2011; Wei et al. 2017) that harbor similar microbial communities (Shi et al. 2014; Lavy et al. 2020) suggesting an important role of bacterial conversion in all these locusts. However, Next Generation DNA Sequencing *S. gregaria* hindgut samples showed considerable variation in bacterial composition between different phases and generations and it is not yet clear to what extent the microbiome is under the control of the locust host, the environment, or locust density (Lavy et al. 2019).

Chemoreception

External Morphology and Types of Sensilla A sensillum is a sensory organ protruding from the exoskeleton of an insect. Different types of sensilla have been described and interpreted as being specialized for the perception of particular types of stimuli: movement, humidity, temperature, smell, and taste known as mechano-, hygro-, thermo-, olfactory- or gustatory- stimulations respectively (Nowińska and Brożek 2017). Current models assume that the external morphology of sensilla can be used to infer their function(s). In a few species the relationship between the morphology of sensilla and what they detect has been explored using information from gene expression or electrophysiological and receptor neuron response experiments (see below), but in general, function is extrapolated from size, shape (Table 1), and location. Sensilla have either no pores (aporous), an apical pore (uniporous), or wall pores (multiporous) (Fig. 3). Those without pores are typically interpreted as mechano-, hygro-, or thermo-receptors, whereas sensilla with pores are considered gustatory (uniporous) or olfactory (multiporous)

Table 1 Types of insect sensilla, their probable function, morphology, and nomenclature

Name	Function	Appearance		Pores	Socket	Length (µm)	Diameter (µm)
Hair or trichoid sensilla[a,b,c]	Mechanical		Long, slender hair-like (see Fig. 3a)	Aporous	Flexible	20–400	3–10
Chaetica or long basiconic sensilla; trichoid sensilla[a,b,d,e]	Mechanical		Peg-like, longitudinal ridges (see Fig. 3b)	Uniporous or aporous		15–300	5–20
Trichoidea or short slender basioconic sensilla[a,b]	Olfactory		Slender hair-like (see Fig. 3e)	Wall-pored (see Fig 3d), fewer than basiconica	Inflexible	5–35	3
Basiconica or short basiconic sensilla[a,b,c]	Olfactory		Stout peg with wavey cuticular depression (see Fig. 3c)	Wall-pored	Inflexible	3–40, typically 10–17	4
	Olfactory		Stout peg-like (see Fig. 3d)	Wall-pored	Inflexible	3–40, typically 9–20	3–4
Coeloconica	Olfactory, thermal		A sharp-tipped peg with longitudinal ridge on the wall, contained within a pit (see Fig. 3f)	Wall-pored	Inflexible	3–10	2–25 pit diameter
	Humidity, thermal		A blunt-tipped peg without grooves, contained within a pit	Apical pore	Inflexible	4–5	2.2–5.5 pit diameter

Sensilla names are based on those established for relatively well-studied short-horned grasshopper, the locust species *Locusta migratoria* and *Schistocerca gregaria* (Altner et al. 1981; Ochieng et al. 1998; Jin et al. 2005; Zhou et al. 2008), but other names used are provided with their sources: a) Bland 1989, b) Chen et al. 2003, c) Li et al. 2007, d) Greenwood and Chapman 1984, e) Chapman 1989

receptors (Nowińska and Brożek 2017). Sensilla are attached with either a flexible or inflexible base (Fig. 3). Sensilla inferred as mechano-sensitive typically have a flexible socket for movement detection, whereas sensilla with inflexible sockets probably detect humidity, temperature, smells, or taste (Nowińska and Brożek 2017). Some sensilla such as those with a flexible socket and an apical pore may have dual functions acting as both movement and taste sensors (Ochieng et al. 1998).

The nomenclature of sensilla types varies depending on species and studies, so standardisation is a first step towards improving the understanding of grasshopper chemoreception

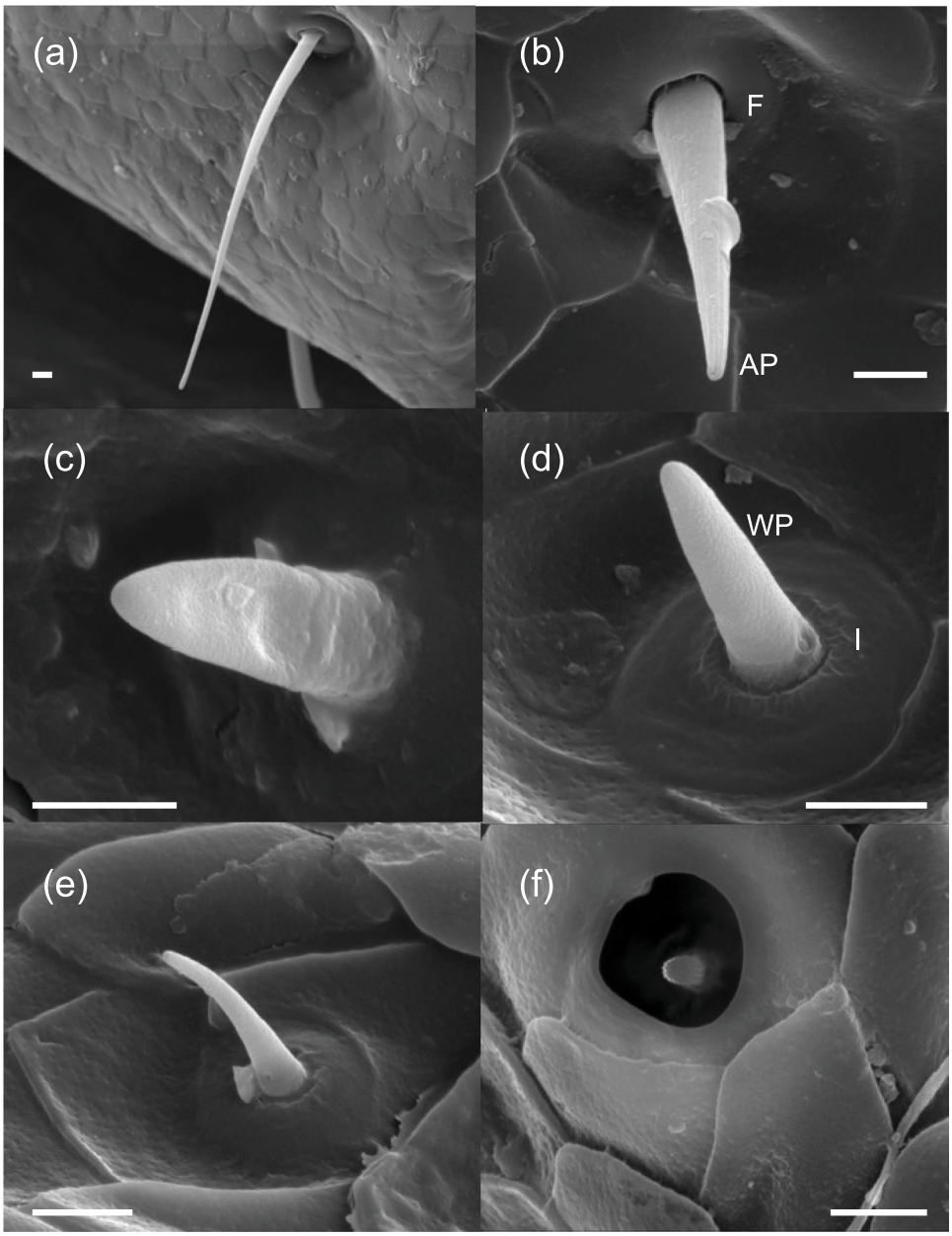


Fig. 3 Sensilla commonly found in Acrididae. **a** Hair sensilla in *Paprides nitidus*, **b** chaetica in *Sigaus australis*, **c** basiconica with wavy cuticular depression in *Brachaspis nivalis*, **d** basiconica in *B. nivalis*, **e** trichoidea in *B. nivalis*, **f** olfactory coeloconica in *S. australis*. F- flexible socket, I- inflexible socket, AP- apical pore, WP- wall-pored. Scale bar 5 µm

(Table 1). Here, we use terminology derived from the study of the locusts *S. gregaria* and *L. migratoria* (Altner et al. 1981; Ochieng et al. 1998; Jin et al. 2005; Zhou et al. 2008). As arable pests, the functions of their sensilla have received the most detailed investigation with physiological (Altner et al. 1981; Ochieng and Hansson 1999) and transcriptomic (Jiang et al. 2017; Jin et al. 2005; Zhou et al. 2008) approaches providing an evidential basis for functional inference. Trichoid sensilla (Fig. 3e) that are responsible for olfaction in locusts, are slender, hair-like sensilla with pores on their walls (Ochieng et al. 1998; Ochieng and Hansson 1999). Much longer than trichoid sensilla with a flexible socket but no pores (Table 1, Fig. 3a) are the hair sensilla responsible for mechanoreception (Bland 1989; Chen et al. 2003; Li et al. 2007; Yu et al. 2011; Zhou et al. 2008). Sensilla chaetica (Fig. 3b) associated with mechano- and gustatory-receptions are thick and peg-like with a flexible socket, ribbed wall, and an apical pore (Bland 1989; Blaney and Chapman 1969; Chen et al. 2003; Jin et al. 2006; Li et al. 2007; Ochieng et al. 1998; Yu et al. 2011; Zhou et al. 2008). Basiconic olfactory sensilla are wall-pored (Fig. 3c and d), but vary in shape among species (Bland 1989; Chapman 1989; Chen et al. 2003; Jin et al. 2006; Li et al. 2007; Ochieng et al. 1998). Sensilla coeloconica consists of a short peg in a cavity commonly considered to be temperature and humidity receptors (Nowińska and Brożek 2017; Jiang et al. 2018). Two types of coeloconica are seen in *L. migratoria*; one with wall pores and one lacking wall pores but with

a single apical pore (Table 1). These sensilla within pits are responsible for detecting smells and temperature, and humidity and temperature, respectively (Altner et al. 1981). The response of wall-pored coeloconica to olfactory stimuli has also been confirmed in *S. gregaria* (Ochieng and Hansson 1999). Wall-pored coeloconica seem to be as common on grasshopper antennae as aporous sensilla (Altner et al. 1981; Bland 1989; Chapman 1989; Chen et al. 2003; Greenwood and Chapman 1984; Li et al. 2007).

Grasshoppers have sensilla on the antennae (Altner et al. 1981; Bland 1989; Chapman 1989; Chen et al. 2003; Greenwood and Chapman 1984; Li et al. 2007; Ochieng et al. 1998), mouthparts (Blaney and Chapman 1969; Chapman 1989; Jin et al. 2006), cerci (Yu et al. 2011), wings (Zhou et al. 2008), and tarsi (Blaney and Chapman 1969; Chapman 1989; Zhou et al. 2009). The relative abundance of particular types of sensilla indicates their function. Sensilla chaetica are abundant on mouthparts (labial and maxillary palps) so they appear to detect gustatory stimuli (Blaney and Chapman 1969; Chapman 1989; Jin et al. 2006), whereas olfactory sensilla including basiconica, trichoidea, and wall-pored coeloconica are more abundant on antennae (Bland 1989; Chen et al. 2003; Li et al. 2007; Ochieng et al. 1998). The proximal end of the antenna (scape and pedicel) is responsible for antennal movements and here mechano-sensilla are abundant (Bland 1989; Chen et al. 2003; Li et al. 2007).

The function of sensilla cannot be inferred purely from morphological examination because many sensilla types vary in shapes and sizes within a type (Bland 1989; Chen et al. 2003; Li et al. 2007; Yu et al. 2011; Zhou et al. 2008, 2009) and the degree to which morphological diversity relates to functional diversity is not known. For example, basiconic sensilla are commonly stout peg-like (either with or without a cuticular depression: Table 1), but other forms have been recorded such as egg-shaped (Chen et al. 2003) or having a peg with an expanded base (Bland 1989; Chen et al. 2003). Subtypes of chaetica are also described in *L. migratoria* according to their size (Yu et al. 2011; Zhou et al. 2009) and the number of neurons connected to them (Jin et al. 2006; Zhou et al. 2009). Incorporating physiological studies such as single sensillum recordings or transcriptomic studies of the expression of receptor protein genes (discussed more in detail later) will help us elucidate the functional diversity of sensilla (Cui et al. 2011; Li et al. 2018; Ochieng and Hansson 1999; Yang et al. 2012).

Sensilla Functional Diversity A variety of pheromones are emitted by insects in various situations (see *Pheromones*) and single sensillum recordings can be used to identify sensillum-specific sensitivity to particular compounds. In the locust *S. gregaria* antennal sensilla were discovered to respond to a variety of odors: basiconica to aggregation pheromones, oviposition attractant, and (*E,Z*)-2,6-nonadienal which is emitted by a preferred host plant (*Tribulus terrestris*) and is potentially a sex pheromone in *S. gregaria*; trichoidea also respond to (*E,Z*)-2,6-nonadienal; and coeloconica to nymph and oviposition pheromones (Ochieng and Hansson 1999). The reason basiconica are capable of detecting multiple olfactory stimuli is likely because they contain as many as 50 olfactory neurons (one to three in trichoidea and coeloconica: Ochieng et al. 1998). Each neuron is potentially responsive to a different odor.

In *L. migratoria*, seven functional subtypes of trichoid sensilla on the antenna have been identified, each housing two to three olfactory neurons (Cui et al. 2011). These neurons responded in several different combinations (i.e., inhibitory vs. excitatory) and intensities to nine different compounds found in frass pheromones (Cui et al. 2011). Some of the compounds found in *L. migratoria* frass pheromones also occur in nymphal (e.g., octanal, hexanal) and aggregation (e.g., guaiacol) pheromones of *S. gregaria* (Ochieng and Hansson 1999) but are detected by basiconica and coeloconica in *S. gregaria* (as mentioned above). Furthermore, trichoid sensilla in *L. migratoria* were discovered to respond to 18 chemicals that were commonly found in their host plants (You et al. 2016) suggesting that trichoid sensilla may be tuned to detect a wider range of compounds in *L. migratoria* than in *S. gregaria*. Together, these studies suggest grasshoppers have a complex pheromone-based communication system that will benefit from further investigation.

Grasshopper Ecology and Sensilla Abundance The number of each type of sensilla on an insect's exoskeleton is linked to the species' ecology. It is thought that high antennal sensitivity results from having a high density of sensilla (Greenwood and Chapman 1984; Bland 1989; Chen et al. 2003). In locusts *S. gregaria* and *L. migratoria*, solitarious individuals have more olfactory sensilla on their antennae than gregarious ones (Greenwood and Chapman 1984; Ochieng et al. 1998). Single sensillum recordings indicate that solitarious *S. gregaria* have a stronger electrophysiological response to some pheromone compounds (e.g., benzaldehyde, acetophenone, and solitarious sex pheromone) than gregarious locusts (Ochieng and Hansson 1999). As the solitarious phase occurs at low population density (Hassanali et al. 2005), higher sensilla abundance in solitarious locusts may reflect a benefit for elevated sensitivity to long-range chemical signals for locating distant conspecifics (Bland 1989; Chen et al. 2003). The number of sensilla may also reflect feeding range (monophagous, oligophagous, or polyphagous) and the heterogeneity of vegetation in the grasshopper's habitat. For example, in Moroccan grasshoppers, polyphagous species (e.g., *S. gregaria* and *Calliptamus barbarus*) have more sensilla on their labrum (about 400-700) than monophagous or oligophagous species (e.g., *Sphingonotus coerulans*, *Oedipoda miniata*; about 200-300

sensilla; Zaim et al. 2013). In another study, desert species *B. argentatus*, *L. coquilletti,* and *C. parviceps* have fewer sensilla (800 – 2000) on their antennae than species living in more equitable habitats such as *Chorthippus curtipennis* and *Metaleptea brevicornis* (4000 – 8500 sensilla; Bland 1989). Species with a limited diet range or desert grasshoppers may be exposed to fewer chemical compounds than species that are polyphagous or living in more complex environments.

Male grasshoppers have more olfactory sensilla on their antennae than females in most grasshopper species studied (80%, n = 75; Chen et al. 2003; Bland 1989; Li et al. 2007). Higher electrophysiological responses to chemical signals in males have also been observed in some of the studies using single sensillum recordings or electroantennography (Ochieng and Hansson 1999; Chen and Kang 2000; Chen et al. 2004). This suggests that males are subject to sexual selection for locating (and possibly discriminating) females (Ritchie 1990). Chinese *Angaracris barabensis* grasshoppers rely on acoustic and visual cues to find mates and there is no sexual difference in sensilla abundance (Chen and Kang 2000). No clear pattern of the sensilla was found among subfamilies or tribes (Bland 1989; Chen et al. 2003; Li et al. 2007; Zaim et al. 2013); and therefore, specific ecological aspects including habitat, feeding patterns and sex roles may influence sensilla abundance and distribution.

Types and Functions of Chemoreceptive Proteins Chemoreceptive proteins are conserved in insects, each type identified by specific amino acid sequences and three-dimensional structures (Sánchez-Gracia et al. 2009). Proteins involved in insect chemoreception are of two distinct types: odorant-binding proteins and chemosensory proteins. These binding proteins are produced in several places in an insect including within sensilla that have chemosensory neurons, where they are secreted in the sensilla lymph (Fig. 4) and transduct chemical signals (e.g., semiochemicals, hormones, nutrients, and toxic compounds) by binding to molecules and moving them to the receptors on the sensory neuron surface (Sánchez-Gracia et al. 2009; Wicher and Miazzi 2021).

Olfactory sensory neurons are nerve cells specialized for transmitting information about smells (Fig. 4). They are equipped with a variety of protein receptors (odorant, ionotropic or sensory neuron membrane) on their dendritic membranes (Sánchez-Gracia et al. 2009) that mediate peripheral neural processing and trigger signals that are transmitted to the brain via ganglia. Odorant receptors are solely responsible for detecting volatiles whereas ionotropic receptors have multiple roles including detection of smells, tastes, humidity, and temperature (Wicher and Miazzi 2021). These receptors have co-receptor(s) for their proper function (Sánchez-Gracia et al. 2009; Cassau and Krieger 2021). Although the exact role of the co-receptors is a mystery, odorant and ionotropic receptors form a complex with their respective co-receptor(s) to function as ligand-gated ion channels (Knecht et al. 2017; Cassau and Krieger 2021). Sensory neuron membrane proteins (SNMPs) are another important membrane protein involved in insect olfaction. There are two main types of SNMPs: co-receptors of pheromone sensitive odorant receptor complex (SNMP1); and proteins expressed in support cells of olfactory sensory neurons (SNMP2) (Cassau and Krieger 2021). The presence of SNMP1 in an olfactory-sensitive neuron and co-expression with odorant receptors show these proteins are involved in the detection of pheromones. Depending on the insect species, there are three (Table 2) or more types (16 in dung beetles) of SNMPs described, although their functions are unknown (Cassau and Krieger 2021).

Functions of Chemoreceptive Proteins Identifying the diversity and function of chemoreceptive proteins involves large-scale genome sequencing, transcription, proteomics, and experimental molecular evolution. Among short-horned grasshoppers these detailed and time-consuming steps have as yet been completed only for *L. migratoria* (Guo et al. 2011, 2020; Wang et al. 2015; Li et al. 2016, 2020; Yu et al. 2016; Chen et al. 2018; Wei et al. 2019). However, the diversity of chemoreceptive proteins has been explored in seven locust species where more olfactory receptor genes are found than ionotropic receptor genes (Table 2). The expansion of olfactory receptors could mean that locusts rely heavily on olfactory cues rather than taste when assessing mates or host plants, whereas in model insect species such as *Drosophila melanogaster*, olfactory receptors, and ionotropic receptors occur in equal numbers (Sánchez-Gracia et al. 2009; Croset et al. 2010).

The potential functions of specific proteins are explored using quantitative real-time PCR and *in situ* hybridization to identify expression in specific tissues, sex, phase, and sensilla. Protein functions can be further inferred by exposing tissues and sensilla to specific chemical stimuli (using electroantennogram or single sensillum recordings) or genome editing tools (Maleszka et al. 2007; Guo et al. 2011; Zhang et al. 2017; Jiang et al. 2021). The genome editing tools RNA interference (RNAi) and Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) have been used on locust species. These techniques allow silencing of the genes that are contributing to discrimination of host and nonhost plants and perception and biosynthesis pheromones and CHCs (Jurado-Rivera et al. 2009; Perkin et al. 2016) by introducing double-stranded RNA (RNAi) or restriction enzymes (CRISPR).

In locusts, olfactory binding proteins and odorant receptors show exclusive or biased expression patterns in antennae and mouthparts suggesting a chemosensory role, whereas chemosensory proteins and ionotropic receptors show broader expression including antennae, abdomen,

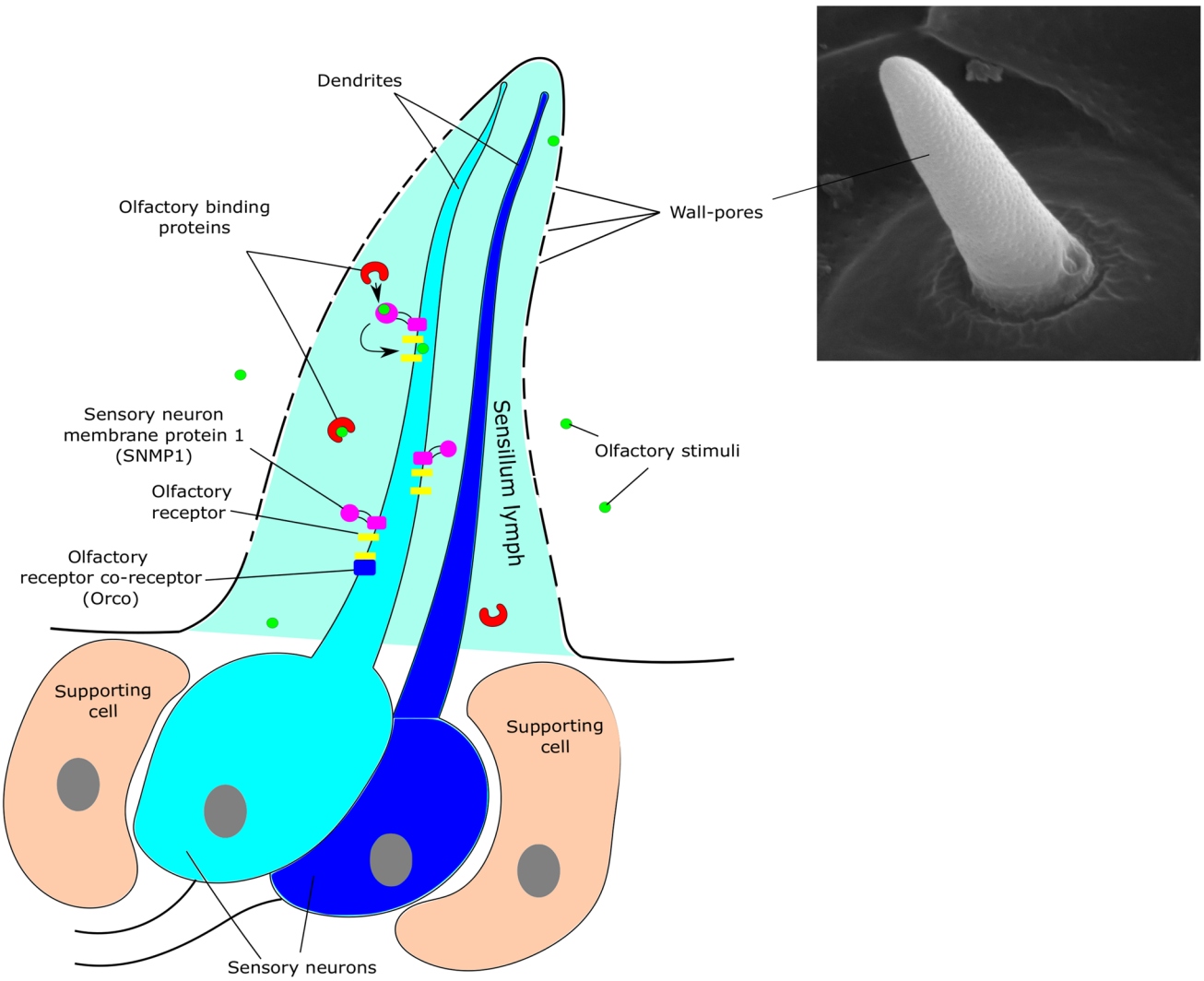


Fig. 4 Simplified scheme of the internal structure of an olfactory sensillum and associated proteins

Table 2 Diversity of proteins involved in chemoreception that bind particles and move them to the receptors in the insect sensilla are shown as numbers of putative functional genes characterized in seven grasshopper species

Name	OBP	CSP	OR	IR	SNMP	References
Ceracris kiangsu Bamboo locust	13	6	91	13	2	Li et al. 2020
Ceracris nigricornis	20	10	71	8	3	Yuan et al. 2019
Locusta migratoria Migratory locust	17	58	142	32	N/A	Wang et al. 2015; Martín-Blázquez et al. 2017; Guo et al. 2018a, 2018b
Oedaleus asiaticus Band-winged grasshopper	15	17	60	6	3	Zhang et al. 2015; Zhou et al. 2019
Oedaleus infernalis	18	N/A	N/A	N/A	N/A	Zhang et al. 2018
Oxya chinensis Rice grasshopper	18	13	94	12	2	Cui et al. 2019b
Schistocerca gregaria Desert locust	14	42	119	>2	2	Guo et al. 2014; Martín-Blázquez et al. 2017; Pregitzer et al. 2017

OBP odorant-binding protein, *CSP* chemosensory protein, *OR* odorant receptor, *IR* ionotropic receptor, *SNMP* sensory neuron membrane protein. The diversity of OR and IR includes its coreceptor(s)

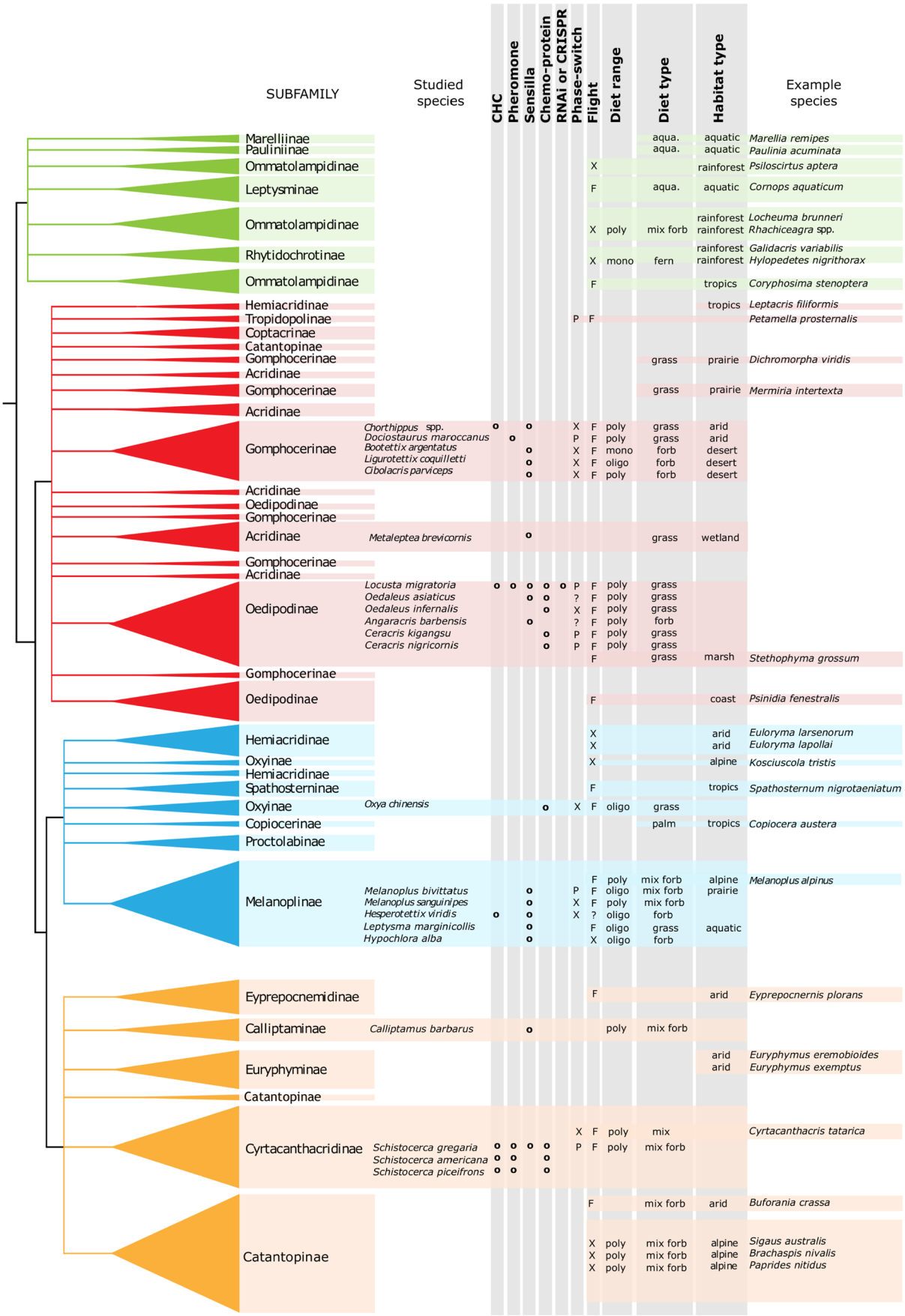
thorax, legs, and wings (Jin et al. 2005; Guo et al. 2011; Wang et al. 2015; Zhang et al. 2018; Cui et al. 2019b; Yuan et al. 2019; Zhou et al. 2019; Li et al. 2020) implying broader functions. Some odorant receptors might be tuned to perceive pheromones, and in insects, odorant receptors are co-expressed with the protein SNMP1 only in pheromone-sensitive neurons (Cassau and Krieger 2021). Co-expression of odorant receptors and SNMP1 in sensilla basiconica and trichoidea of *S. gregaria* (Pregitzer et al. 2017) are responsible for detecting an aggregation pheromone and a putative sex pheromone respectively (Ochieng and Hansson 1999). In *L. migratoria*, specific odorant receptors expressed in sensilla basiconica are responsible for detecting aggregation pheromone (Guo et al. 2020). The co-receptor of an odorant receptor plays a crucial role in insect olfaction, and laboratory silencing of this protein in *L. migratoria,* using both RNAi (Wang et al. 2015) and CRISPR/Cas9 (Li et al. 2016), resulted in the loss of sensitivity to pheromones and food plant odors. Wang et al. (2015) also found that two co-receptors of ionotropic receptors were silenced but this did not affect the sensitivity of the grasshopper to the pheromone, suggesting reception may be specific to odorant receptors. Furthermore, odorant receptors are also expressed in trichoidea, which are responsible for perceiving plant-derived chemicals (ketones and esters) (You et al. 2016) and frass pheromones (Cui et al. 2011). These studies show odorant-receptor families respond to both insect- and plant-derived olfactory stimuli. Ionotropic receptors also respond to some chemical stimuli. In *S. gregaria*, some ionotropic receptors are expressed in sensilla chaetica and coeloconica (Guo et al. 2014). Coeloconica are tuned to detect odors of food plants (Ochieng and Hansson 1999) and chaetica are considered to be taste receptors (Ochieng et al. 1998). Thus, it is possible that these ionotropic receptors are responsible for detecting a range of chemical cues from food plants. Whether or not ionotropic receptors are involved in other roles (e.g., temperature and humidity perceptions) is not known for locusts.

Sex-biased (Wang et al. 2015; Zhang et al. 2015, 2018; Cui et al. 2019b; Yuan et al. 2019; Zhou et al. 2019; Li et al. 2020) or phase-biased (Guo et al. 2011) variation in the amount produced of some proteins indicates they are used to mediate chemosensory processing that is specific to sex (e.g, males to perceive female pheromone; females to locate oviposition site) or phase. In *L. migratoria*, one of the chemosensory proteins showed significantly higher expression in gregarious nymphs compared to solitarious ones, and RNAi-mediated silencing showed this chemosensory protein was involved in an attraction response to an aggregation pheromone (Guo et al. 2011). Sex-biased or -specific expression of some odorant-binding proteins, chemosensory proteins, odorant receptors, and ionotropic receptors has been observed in several locust species (Table 2), suggesting they mediate sex-specific chemosensory processing (e.g., males to perceive female pheromone; females to locate oviposition sites).

Applied Chemical Ecology in Pest Control and Conservation

Knowledge of the chemical ecology of insect species presents an opportunity to reduce reliance on insecticides, which present major concerns for environmental resilience, biodiversity, human health, and the development of insect resistance to insecticides (Giron et al. 2018). The application of genome editing tools has recently provided new opportunities for pest control and may be important for reducing the devastation that results from locust plagues. The shift to the swarming phase of locusts is controlled by pheromone signaling and is accompanied by rapid change in their olfactory pathways (Hassanali et al. 2005). Thus, identification and knockdown of the genes involved in the pheromone synthesis or pheromone-reception of the gregarious phase have been the primary interest of chemical ecology studies of locust species. So far, only the genome of *L. migratoria* has been edited in this way. Genes involved in the perception (Guo et al. 2011, 2020; Ma et al. 2015, 2019; Li et al. 2016; Zhang et al. 2021) and biosynthesis of aggregation pheromones (Wei et al. 2019) and CHCs (Wu et al. 2020; Zhang et al. 2021) have been successfully silenced using RNAi and CRISPR/Cas9. RNAi interferes with existing gene expression but CRISPR results in permanent genetic modification; thus, higher stability and the potential to pass genetic changes to their offspring. Moreover, CRISPR along with gene-drive has the potential to help spread the manipulated alleles in the field more rapidly than via normal Mendelian processes (Giron et al. 2018; Courtier-Orgogozo et al. 2020). The stability of transformed genes in natural populations is not known and could be affected by the presence/absence of resistant strains (Sugahara et al. 2017) and natural selection may reduce its effectiveness. There are unknown but potentially serious implications of the spread of modified genetics to non-target species through hybridization and/or horizontal transfer via vectors such as mites, parasitoids, viruses, or microsporidia (Sugahara et al. 2017; Giron et al. 2018; Courtier-Orgogozo et al. 2020). Risk assessment of the genetic spill-over using mathematical models is a current focus of gene editing and gene drive (Courtier-Orgogozo et al. 2020; Greenbaum et al. 2021). Studies using experimental evolution under controlled and semi-controlled conditions are also required before applying this method in the field.

Although the application of genome editing tools in pest control is increasing, the efficacy of artificially modified


SUBFAMILY
Studied species
CHC
Pheromone
Sensilla
Chemo-protein
RNAi or CRISPR
Phase-switch
Flight
Diet range
Diet type
Habitat type
Example species
Marelliinae
Paulinilinae
Ommatolampidinae
Leptysminae
Ommatolampidinae
Rhytidochrotinae
Ommatolampidinae
Hemiacridinae
Tropidopolinae
Coptacrinae
Catantopinae
Gomphocerinae
Acridinae
Gomphocerinae
Acridinae
Gomphocerinae
Chorthippus spp.
Dociostaurus maroccanus
Bootettix argentatus
Ligurotettix coquilletti
Cibolacris parviceps
Acridinae
Oedipodinae
Gomphocerinae
Acridinae
Metaleptea brevicornis
Gomphocerinae
Acridinae
Oedipodinae
Locusta migratoria
Oedaleus asiaticus
Oedaleus infernalis
Angaracris barbensis
Ceracris kigangsu
Ceracris nigricornis
Gomphocerinae
Oedipodinae
Hemiacridinae
Oxyinae
Hemiacridinae
Spathosterninae
Oxyinae
Oxya chinensis
Copiocerinae
Proctolabinae
Melanoplinae
Melanoplus bivittatus
Melanoplus sanguinipes
Hesperotettix viridis
Leptysma marginicollis
Hypochlora alba
Eyprepocnemidinae
Calliptaminae
Calliptamus barbarus
Euryphyminae
Catantopinae
Cyrtacanthacridinae
Schistocerca gregaria
Schistocerca americana
Schistocerca piceifrons
Catantopinae
aqua.
aqua.
rainforest
aquatic
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desert
desert
grass
wetland
grass
grass
grass
grass
grass
grass
marsh
coast
arid
arid
alpine
tropics
tropics
alpine
prairie
aquatic
arid
arid
arid
mix
mix forb
mix forb
arid
alpine
alpine
alpine
Aquatic Marellia remipes
Aquatic Paulinia acuminata
Rainforest Psiloscirtus aptera
Aquatic Cornops aquaticum
Rainforest Locheuma brunneri
Rainforest Rhachiceagra spp.
Rainforest Galidacris variabilis
Rainforest Hylopedetes nigrithorax
Tropics Coryphosima stenoptera
Tropics Leptacris filiformis
Petamella prosternalis
Prairie Dichromorpha viridis
Prairie Mermiria intertexta
Arid
Arid
Desert
Desert
Desert
Wetland
Marsh Stethophyma grossum
Coast Psinidia fenestralis
Arid Euloryma larsenorum
Arid Euloryma lapollai
Alpine Kosciuscola tristis
Tropics Spathosternum nigrotaeniatum
Tropics Copiocera austera
Alpine Melanoplus alpinus
Prairie
Aquatic
Arid Eyprepocnemis plorans
Calliptamus barbarus
Arid Euryphymus eremobioides
Arid Euryphymus exemptus
Cyrtacanthacris tatarica
Schistocerca gregaria
Schistocerca americana
Schistocerca piceifrons
Arid Buforania crassa
Alpine Sigaus australis
Alpine Brachaspis nivalis
Alpine Paprides nitidus

◂**Fig. 5** Our current knowledge of Acrididae chemical ecology is patchy, as revealed by examination of its extent in a phylogenetic context (derived from Song et al. 2018). Grasshopper biological traits (phase switch, flight/flightless, diet range, diet type, and habitat) of best studied species are indicated. Availability of information on cuticular hydrocarbon (CHC), pheromone, sensilla, chemo-protein and/or genome editing tools (RNAi or CRISPR) is indicated (o indicates the species has been studied). Exemplars of the broader, unstudied, ecological diversity of the Acrididae is indicated on the right. P- capable of phase-switching, F- capable of flying, X- incapable of phase-switching or flying, poly- polyphagous, oligo- oligophagous, mono- monophagous, mix forb- feeding on both grasses and forbs but prefer forbs more

individuals may be reduced if plant-insect and insect-insect interactions are altered in natural populations of locusts by factors associated with climate change. Changes in irradiation intensity, temperature, CO_2 concentration, and humidity are known to affect the chemical composition (nutrients and secondary metabolites) of plants by altering biosynthetic pathways, and emission quantity and frequency by modifying stomatal opening/closing (where volatiles are predominantly emitted) (Giron et al. 2018; Effah et al. 2020). As feeding, oviposition, and development of locusts (and likely other short-horned grasshoppers) respond to plant-derived chemical signals (discussed in *Plant-derived Signals*), alteration of plant chemical signals could influence plant-insect interactions. Changes in host plant-derived chemicals may also alter symbiotic microbiome composition since certain bacteria contribute to locust pheromone signals (e.g., guaiacol and phenol: see *Chemical Biosynthesis in Grasshoppers*), and play an important role in resistance against pathogens and parasites (Lavy et al. 2020). Moreover, an increase in ambient temperature can increase body temperature in insects, which in turn, affects biosynthetic pathways in insects (Giron et al. 2018). This can alter locust chemical communication as some aggregation pheromones (i.e., guaiacol and phenol) are reliant on bacterial conversion. Humidity levels probably exert strong selection pressure on the chemical composition of their cuticular hydrocarbons (as discussed in *Cuticular Hydrocarbons (CHCs)*).

In addition to genome-editing tools, entomopathogenic microorganisms have also been considered for insect pest management. Entomopathogenic microorganisms are biopesticides, impairing chemical signaling, reproduction, and mobility of their hosts. Some are acridid-specific (e.g., *Metarhizium anisopliae var. acridum*: Atheimine et al. 2014; Abdellaoui et al. 2020; *Paranosema locustae*: Shi and Njagi 2004; Shi et al. 2014), which suggests a limited effect on non-target organisms. The gut microsporidian parasite, *P. locustae* has been observed in *L. migratoria* (Shi and Njagi 2004; Shi et al. 2014) and found to disrupt chemical communication in the locusts by reducing olfactory sensitivity (Shi and Njagi 2004), preventing biosynthesis of bacterial mediated pheromones, and lowering activities of neurotransmitters (serotonin and dopamine) that are required for initiation and maintenance of gregarious behavior (Shi et al. 2014). Furthermore, the effectiveness of dry coinidia (fungal spores) of the entomopathogenic fungus *M. anisopliae var. acridum* on 4th instar *S. gregaria* was apparent at varying vegetation composition, temperatures (23-48 °C), and relative humidity (4-52%) (Atheimine et al. 2014), suggesting potential to remain useful in a changing climate. In comparison to chemical pesticides, both genome editing and entomopathogenic techniques are relatively new and their use in pest control is still developing. Studies relating to these tools are accumulating and considered to be promising alternatives to insecticides. A better understanding of chemical ecology and chemoreception of insects as well as chemical changes in plants associated with climate change can aid our development of both pest control tools and species conservation strategies.

Future Directions

Short-horned grasshoppers are fascinating subjects for the study of chemoreception and chemical ecology by virtue of their taxonomic and ecological diversity (Fig. 5). The use of chemical signals has been inferred for a variety of short-horned grasshopper species but, not surprisingly, most chemical ecology studies are focused on the economically important locust pest species (Fig. 5). Locust species exhibit similar ecology and structures including strong flight, phase-switching, chemical signaling (e.g., aggregation pheromones), and diversity of chemoreceptive proteins (i.e., greater diversity of odorant receptors compared to ionotropic receptors), yet they do not form a monophyletic group. A locust is therefore a converged strategy that has independently evolved in four subfamilies; Gomphocerinae, Oedipodinae, Melanoplinae, and Cyrcanthacridinae (Fig. 5). As such, our understanding of the convergence of signaling traits will be enhanced by studies of their phylogenetic relatives. Mechanisms of chemoreception (sensilla), chemical-mediated behaviors, diversity of expression patterns, and specific functions of chemoreceptive proteins in locusts have provided an invaluable framework for the study of the Acrididae more widely. Future research involving species that represent the taxonomic breadth of the family and the range of biological traits that they display (e.g., flightless, monophagous, alpine, aquatic, rainforest) could reveal evolutionary constraints on chemoreception and chemical ecology. Several strands of research would be illuminating: 1) The morphological diversity of sensilla in a variety of species can be explored using SEM, and the functional diversity of specific sensilla types could be further investigated using single sensillum recordings or chemoreceptor-deorphaning approaches. It would be extremely valuable to know whether

the function of a sensillum can be accurately inferred from its external characters. 2) The genetic and functional diversity and expression patterns of chemoreceptive proteins of acridids other than locusts require further research using genome sequencing, transcriptomics, and molecular evolution approaches. 3) The use of short- (CHCs) vs. long- (volatile) distance chemical signals could be elucidated using a combination of analytical tools, behavioral assays, and electrophysiology. The potential for using pheromones to control grasshopper species that damage crops is likely to be an economically important line of research. 4) Exploring the biosynthetic pathways and the role of catalysts and gut bacteria in the synthesis of pheromones would be valuable. Determining the host-specificity of gut microbes in grasshoppers, their method of transmission, and potential for host switching and transferring chemical signaling phenotypes between species is an exciting area of current research. Manipulating insect physiology by feeding them bacteria is one avenue of pest management likely to reduce insecticide use. The interactions of microbes and their grasshoppers can be revealed using a range of experimental tools, biochemical and -omics approaches (metagenomics, transcriptomics, and metabolomics). Integrated research involving these tools could enhance our understanding of the evolution of chemoreception and chemical ecology in the Acrididae, with implications for species interactions, speciation, conservation, pest management and resilience to environmental change (e.g., Dyer et al. 2018; Mori and Noge 2021; Wang et al. 2021).

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Code Availability (Software Application or Custom Code) Inkscape 1.0.

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Declarations

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In which chapter is the manuscript/published work?	3
What percentage of the manuscript/published work was contributed by the student?	75%
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Chapter 3

A comparative approach to understand the food plants of three species of New Zealand Alpine Grasshoppers

3.1 Introduction

Under ecological theory species that are similar in their use of resources are less likely to coexist, however many generalist herbivores such as grasshoppers occupy the same habitat and seem to coexist despite using essentially the same resources (Kaplan and Denno 2007; Behmer and Joern 2008). The range of plants eaten by each herbivore species within a resource-partitioning framework might involve differences in proportions or tissue types rather than species eaten, or micro-habitat partitioning (Behmer and Joern 2008). This chapter focuses on diet, but the availability of food is only one factor influencing species coexistence. Others factors such as access to oviposition sites, climatic variation etc and indirect biotic interactions such as parasites and predators might also limit abundance and reduce competition to explain coexistence (Kaplan and Denno 2007).

Although the majority of short-horned grasshoppers (Acrididae) eat more than one type of plant (oligophagous or polyphagous; Joern 1979; Behmer and Joern 2008; Ibanez et al. 2013), animals are selective feeders, eating a subset of available plant species (Joern 1979b; Ibanez et al. 2013a). Among grasshoppers species that have been studied, some appear to feed only on herbaceous dicots (forbivorous) or only on monocots (graminivorous), whereas others are mixed feeders (ambivorous) but may have a preference for dicots or grasses (Joern 1979b; Ibanez et al. 2013a; McClenaghan et al. 2015). In addition to selecting among available plant species, different parts of particular plants are consumed (Watson 1970; White and Watson 1972).

In the New Zealand alpine zone, the grasshoppers *Brachaspis nivalis*, *Sigaus australis* and *Paprides nitidus* co-occur across much of their spatial distribution. In the Craigieburn Range, these grasshoppers are known to feed on more than 100 plant species present in the area

(Watson 1970) with a preference for dicots over monocots (Supplementary Figure 3.1; Supplementary Table 3.1).

Insect mandible morphology reflects adaptation to broad food types (Krenn 2019). Pronounced ecological diversity is present including the proboscis of adult Lepidoptera which is a nectar-feeding device giving access to long-tubed flowers and the hair-like structures on mouthparts of filter-feeding aquatic insects (larvae) that trap particles suspended in water. Grasshoppers are biting and chewing insects feeding primarily on plant tissue (Clissold 2007; Krenn 2019) and examination of the mandibles of North American (Isely 1944; Gangwere 1965), Asian (Kang et al. 1999), and African (Chapman 1964) grasshoppers reveals two morphological types: predominantly graminivorous grasshoppers with blunt incisors and molars (Figure 3.1A) and forbivores with sharp incisors and molars (Figure 3.1B–D). These mandibular forms may reflect physical differences among the leaves of dicots and grasses, such as leaf toughness (Clissold 2007; Ibanez et al. 2013a) and leaf venation (Clissold 2007). Grasshopper mandibles have heavily sclerotized surface cuticle (Figure 3.1) due to impregnation with melanin or heavy metals (e.g., zinc, manganese, or iron) (Krenn 2019) and so are resistant to wear.

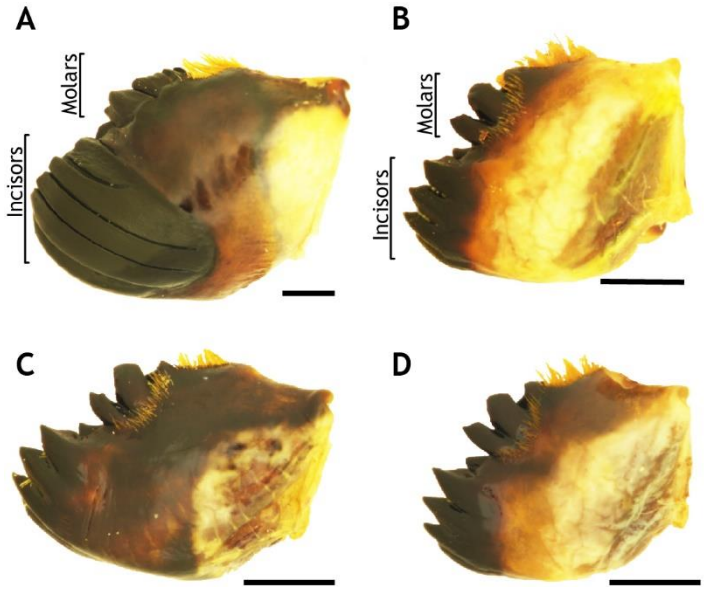

Figure 3.1 Mandibles of grasshopper species have different proportions of melanization that might be adaptations to different food plants or plant types. (**A**) cosmopolitan locust *Locusta migratoria*, and three endemic, sympatric New Zealand alpine grasshoppers; (**B**) *Paprides nitidus*, (**C**) *Sigaus australis*, (**D**) *Brachaspis nivalis*. External surface of left mandible of females individuals. Scale bar = 1mm.

Grasshoppers exhibit sexual size dimorphism where adult females are larger than adult males (Bigelow 1967; Patterson 1983; Vincent 2006; Meza-Joya et al. 2022), and this may result in food use differences between them. For example, a positive correlation between head size and plant foliage length, width and thickness was observed in the Eastern lubber grasshopper *Romalea microptera*, such that food plants of different species (and foliage traits) contribute different proportions of male and female diet (Vincent 2006). Therefore, measuring functional traits of grasshoppers may allow us to elucidate whether males and females and sympatric species have adaptions for different plant foods.

Quantifying a herbivore's diet is challenging and typically a combination of approaches is most revealing. Direct observations of foraging and feeding provide compelling insight about behaviour and diet (e.g. Watson 1970; Ibanez et al. 2013; Mallott et al. 2018) but only represent brief windows on feeding. Less direct approaches can provide a better overview of dietary range by examining the plant materials consumed via microhistological epidermal

analysis (e.g., Watson 1970; Joern 1979; Trewick 1996; Soininen et al. 2009) or DNA metabarcoding of gut content or frass/faeces (e.g. Jurado-Rivera et al. 2009; Soininen et al. 2009; McClenaghan et al. 2015; Mallott et al. 2018; Welti et al. 2019). The chloroplast genes ribulose-1,5-bisphosphate carboxylase-oxygenase (*rbc*L also known as RuBisCo, rubisco, RuBPCase, or RuBPco) and an intron of transfer RNA leucine (*trn*L) are commonly sequenced as primers for these chloroplast regions are able to amplify a wide range of plant taxa making them useful for plant DNA metabarcoding (McClenaghan et al. 2015; Mallott et al. 2018).

Here, I investigated mandible morphology of sympatric adult males and females of three grasshopper species and gathered data on the plant species they consumed using epidermal analysis and amplicon sequencing. I predict that males and females of three alpine grasshopper species would diverge in mandible morphology and diet. I also evaluated the precision and taxonomic resolution of DNA metabarcoding between *rbc*L and *trn*L.

3.2 Materials and Methods

Adult grasshoppers of *Brachaspis nivalis*, *Sigaus australis* and *Paprides nitidus* and reference plant materials were collected from the mountains in central South Island (and North Island in case of some plant specimens) for mandible and gut content analyses (Figure 3.2). Grasshopper specimens used for mandible analysis and gut content analysis were collected at different years and sites (Supplementary Table 3.2). Gut content analysis includes DNA and microhistological epidermal analysis, and different gut sections were used for each analysis (Figure 3.2).

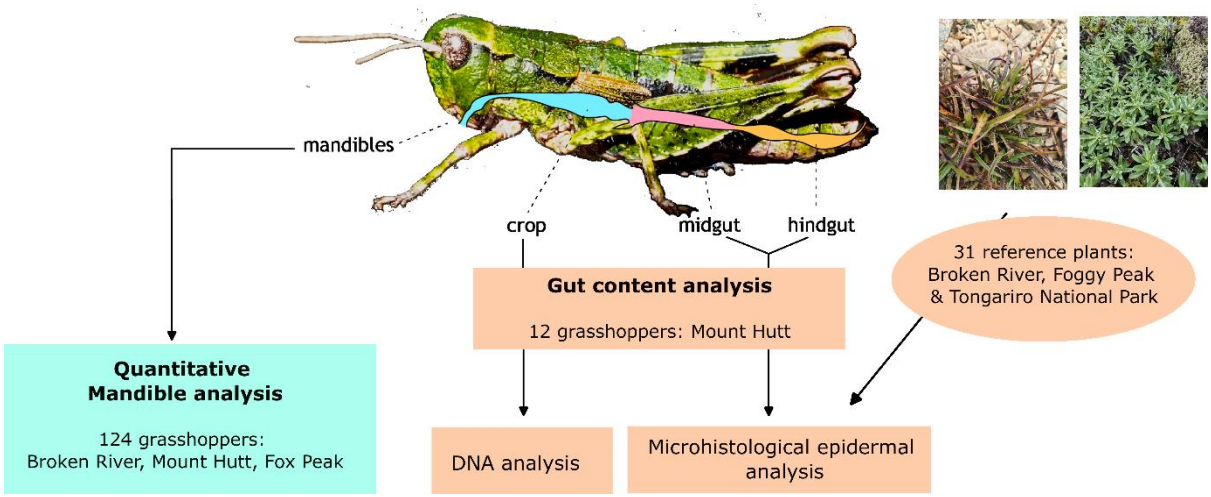

Figure 3.2 Collection sites and sample size of grasshopper and reference plant specimens used for mandible and gut content analyses. Detailed sample sizes of grasshopper specimens and a list of plant species collected are in Supplementary Tables 3.2 & 3.3.

Mandible morphological measurements and statistical analysis

Grasshopper specimens used for mandibular analysis were from the Phoenix Lab collection (Massey University) collected from Mount Hutt Ski Area (-43.5118, 171.5492) and Fox Peak Ski Area (-43.8530, 170.8077) in February 2016 (authorization number: 49878-RES) and additional specimens collected from Broken River Ski Area (-43.125750, 171.686239) in February 2021 (authorization number: 97397-FLO) with authority from the New Zealand Department of Conservation and ski area operators. Between 16 and 24 ethanol preserved

grasshoppers of each sex and species were used for the mandible analysis. The left mandible of each specimen was excised and three linear measurements made between four homologous points based on studies by Patterson (1983, 1984): incisive length (length from posterior articular process to the tip of the second incisor), molar length (length from the articular process to the tip of the second molar) and articular hinge length (length between the posterior and anterior processes) (Figure 3.3). Measurements were performed using a Leica stereo microscope (SM225, Olympus, Japan) equipped with a digital camera (SC180, Olympus, Japan) and imaging software (NIS-Elements 5.01, Nikon Instruments Inc., USA). For consistency, measurement points were aligned under the microscope in the same horizontal plane by adjusting the points in focus. Each measurement was taken two or three times on different days and the average was used as the final measurement.

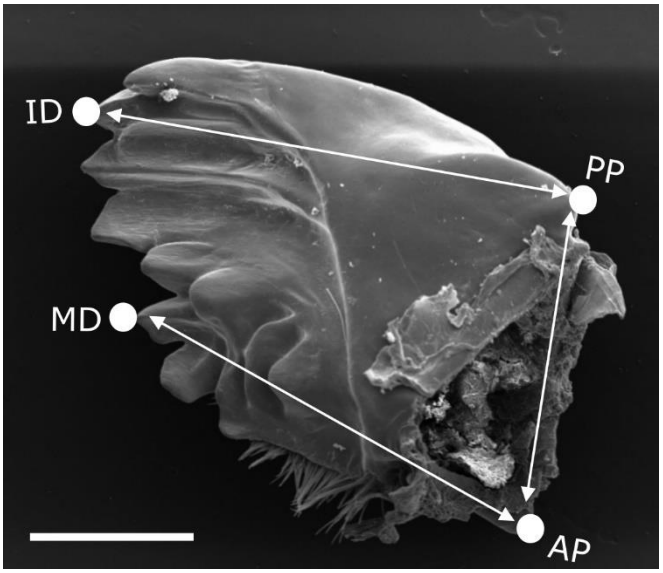

Figure 3.3 Internal view of the left mandible from female *Brachaspis nivalis* grasshopper. ID = incisor dents, M = molar dents, PP = posterior articular process, AP = anterior articular process (Patterson 1983, 1984). Articulation hinge (PP to AP), incisor (PP to ID) and molar (AP to MD) lengths were measured. Scale bar = 1mm.

To analyse the area of melanization on the alpine grasshopper mandibles, the anterior surface of the left mandible was photographed by mounting on double-sided tape (OfficeMax Hook & Loop Fastners Spot White 22mm). Images of the mandibles were acquired with a Leica stereo microscope (SM225, Olympus, Japan) equipped with a digital camera (SC180, Olympus, Japan) with consistent illumination for all specimens. To avoid reflections, a cylindrical (22mm diameter, 20mm height) paper towel diffuser was placed around the specimen (Figure 3.4A).

ImageJ (LOCI, University of Wisconsin) was used to threshold and analyse the images as follows (Figure 3.4B–D). Firstly, the image was converted into an 8-bit grey-scale (Image >Type > 8-bit; Figure 3.4B). Then, the area of interest was selected (with polygon selection; Figure 3.4C), using homologous landmarks present in all species and sexes of the grasshoppers. The brightness threshold was set 0 as minimum and 100 as maximum (0 = pure black and 255 = pure white) using the 'Threshold' function (Image > Adjust > Threshold; Figure 3.4D). Finally, using the 'Analyze Particles' function (Analyze > Analyze Particles), the proportion (%) of area of interest (yellow line in Figure 3.4D) that was in the range of the threshold (red colour in Figure 3.4D) was calculated. This method was based on Siegenthaler et al. (2017) with some modifications to the functions used.

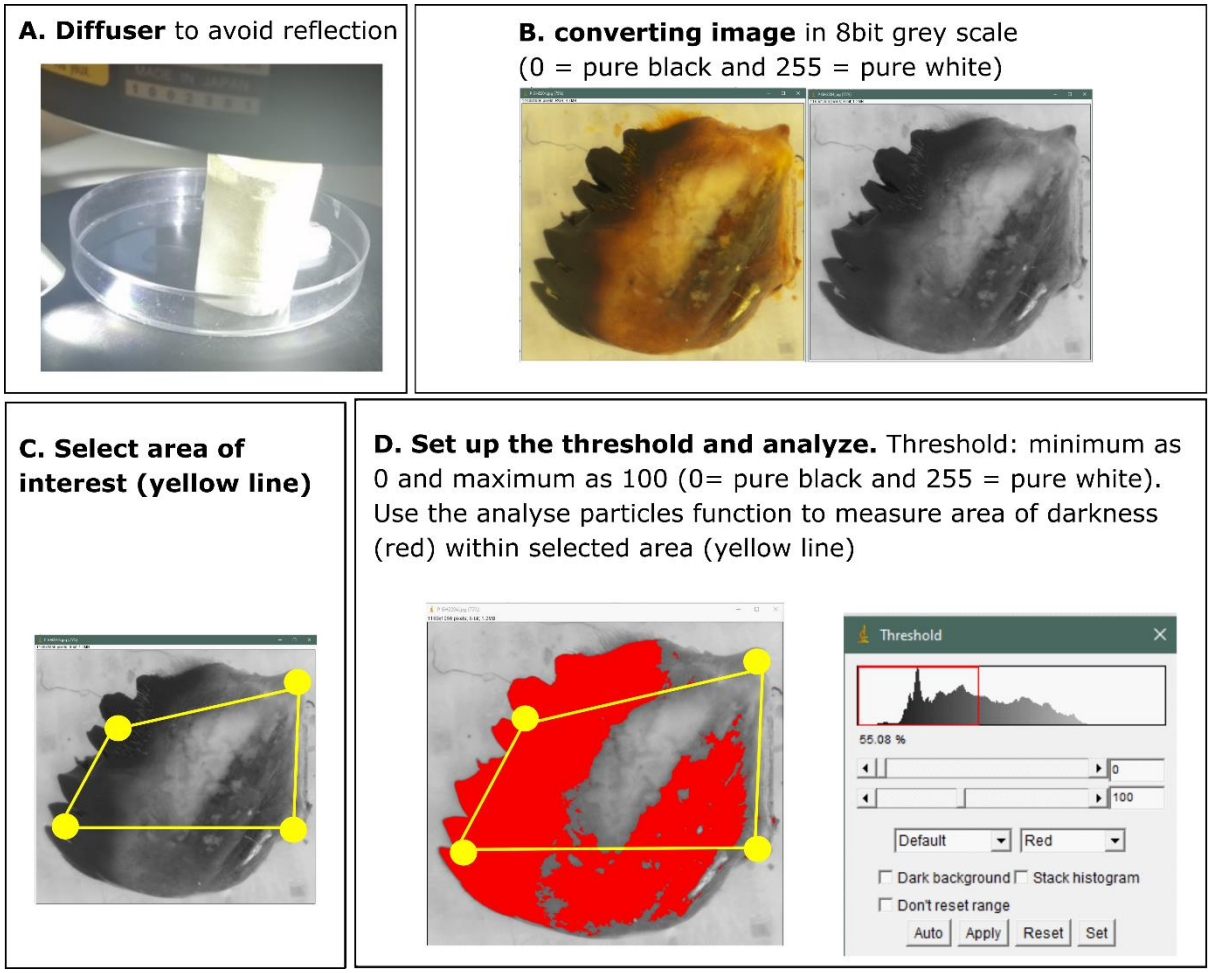

Figure 3.4 Protocol for measuring melanized area of New Zealand alpine grasshoppers. **A** shows diffuser wrapped around the specimen to avoid reflection; **B–D** outline the steps performed in ImageJ.

All statistical analyses were performed in the R statistics environment (R Core Team 2023) using the software platform R Studio 4.0.3 (Boston, MA, USA) and graphics are generated using R Studio and Inkscape 1.2. The statistical normality of data sets was tested using Shapiro-Wilk test. A principal component analysis (PCA) was performed on four mandibular variables (incisor, molar and articulation hinge length and area of melanization) and 60 individuals using 'factoextra' function in R. Differences in incisor, molar and articulation hinge lengths and area of melanization among species and sexes of the grasshoppers were estimated by a linear model, followed by a post hoc Tukey honest significant differences (Tukey HSD) for multiple comparisons.

Gut content analysis

Plant epidermal analysis

For analysis of gut content, 12 adult grasshoppers (n=2 per sex per species) were collected at Mount Hutt Ski Area at 1600 m.a.s.l (-43.495870, 171.539220) in February 2022 (authorization number: 97397-FLO) within a small (20 m^2) patch of habitat (Figure 3.5) to minimize confounding variation in vegetation available to them. Each specimen was preserved in 99% ethanol upon collection with incised abdomen to maximize preservation of DNA in gut contents.

Figure 3.5 Habitat patch near Mount Hutt Ski Area (-43.495870, 171.539220), where *Brachaspis nivalis*, *Paprides nitidus* and *Sigaus australis* were collected for gut content analysis.

Midgut and hindgut contents were dissected from grasshoppers and cleaned in bleach for 10 minutes. Plant fragments were washed with water and 70 % ethanol, spread on a slide, and stained with basic fuchsin stain gel (water 60 g, glycerin 17.5 g, gelatin 10 g, Listerine 2.5 g). Reference collection of epidermal tissue mounted on microscope slides was prepared for 31 plant species collected from Broken River Ski Area (-43.125750, 171.686239) in 2021 and

Tongariro National Park (-39.197260,175.564351) and Foggy Peak (-43.294107,171.744770) in 2022 (Supplementary Table 3.3). The plants chosen were previously shown to be important elements in the diet of these grasshopper species (Watson 1970) and preparation followed García-Gutiérrez et al. (2020).

Microhistological studies use tissue morphology traits including the distribution, orientation, shape and size of epidermal cells and stomata to identify the source of plant fragments (Da Silva et al. 2016; García-Gutiérrez et al. 2020). Leaves of monocots (Figure 3.6A–F) and elongated plant structures (e.g., stems and petioles) have elongated epidermal cells that are aligned in parallel. In contrast, the epidermal cells of the leaves of dicots (Figure 3.6I–X) and ferns (Figure 3.6G & H) have a range of shapes and arrangements (Evert 2006). Cell morphology often differs between adaxial (Figure 3.6R & S) and abaxial surfaces of a leaf (Figure 3.6T) as well as between edge (Figure 3.6S) and middle (Figure 3.6R).

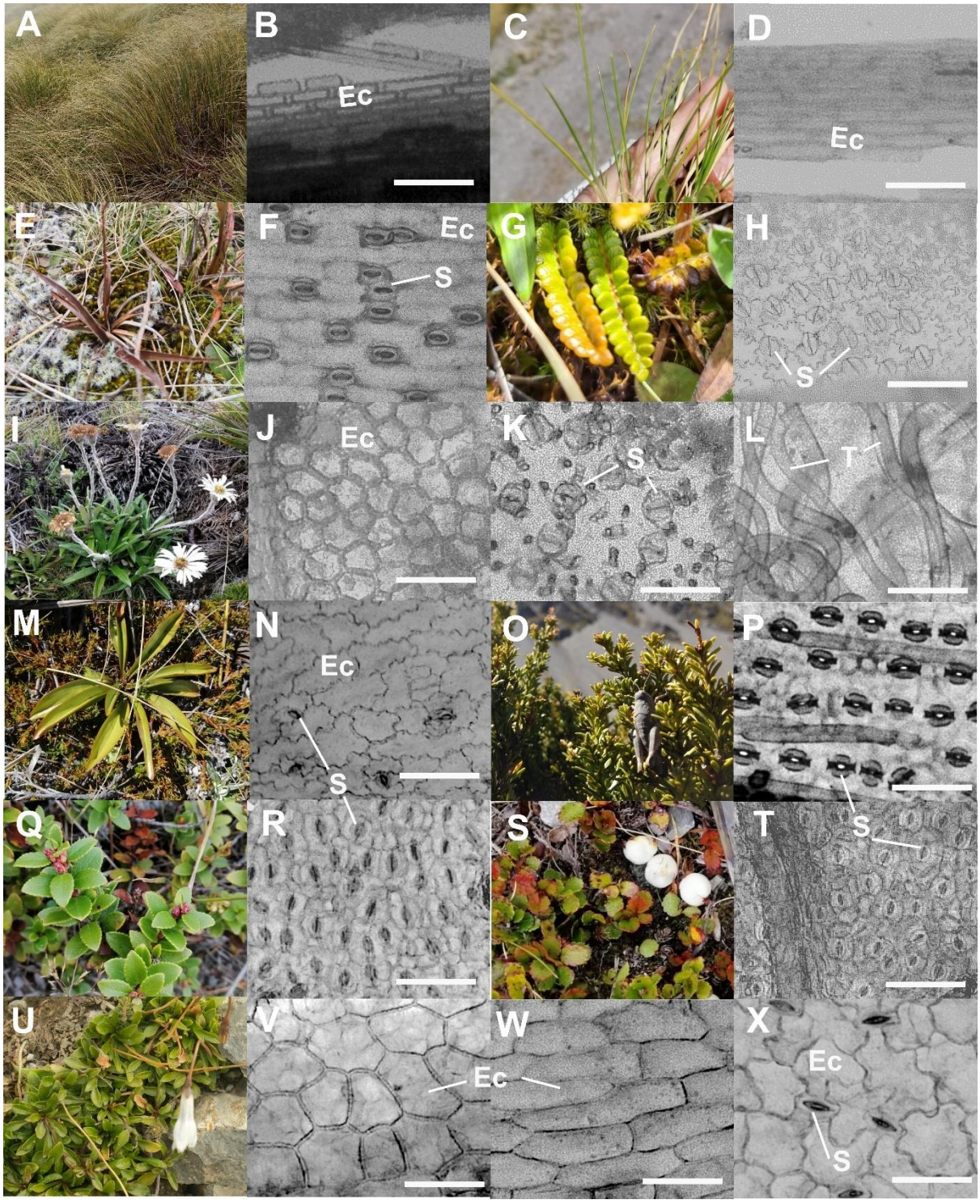

Figure 3.6 Examples of New Zealand alpine plants and their leaf epidermal morphology: **A, B** *Chionochloa* spp.; **C,D** *Poa colensoi*; **E,F** *Luzula* spp.; **G,H** *Blechnum penna-marina*; **I–L** *Celmisia spectabilis*; **M,N** *Gentianella corymbifera*; **O,P** *Podocarpus nivalis* (and *Brachaspis nivalis*); **Q,R** *Gaultheria crassa*; **S,T** *Gaultheria depressa*; **U–X** *Wahlenbergia albomarginata* (**V,W** adaxial; **X** abaxial surface). Scale bars = 200 µm. Abbreviations: S = stoma(ta), Ec = epidermal cell, T = trichomes.

DNA extraction and sequencing

Grasshopper crop contents were dissected out of each insect and DNA extracted using the GeneJET PCR Purification Kit (Thermo Fisher Scientific) according to the manufacturer's instructions. To determine that the DNA extractions were suitable for downstream analysis dilutions were used to trial plant-specific PCR primers. The *rbcL* DNA barcoding primers were, forward CTTACCAGYCTTGATCGTTACAAAGG (Erickson et al. 2017) and reverse GTAAAATCAAGTCCACCRCG (Kress and Erickson 2007). These have an expected fragment size of 379 base pairs (Erickson et al. 2017). For *trn*L I used *trn*L-c A49325 (forward) CGAAATCGGTAGACGCTACG; and *trn*L-d B49863 (reverse) GGGGATAGAGGGACTTGAAC; Taberlet et al. 2007) which are expected to yield a fragment of approximately 450 bp, although INDELS within the *trn*L intron result in length variation among species (Taberlet et al. 2007). The *trn*L and *rbc*L regions of the chloroplast were amplified as follows: 95^{o}C for 1 min, 34 cycles of 30sec at 94 °C, 30sec at 50 °C and 1 min at 72 °C with a final extension of 10 min at 72 °C. The PCR products were visualized on a 1 % agarose gel staining with SYBR^{TM} green. DNA of sufficient quality for successful amplification of cpDNA fragments was sent for amplicon DNA sequencing on NovaSeq using 250 base pair (bp) Paired-Ends (PE).

DNAs from the contents of crops of 12 grasshoppers were subject to polymerse chain reaction amplification for the two chloroplast targets *rbc*L and trnL. After initial laboratory trials to confirm their suitability, amplification with customised primers, barcode and library preparation, and library QC was done by Custom Science Ltd (www.customscience.co.nz). DNA sequencing was on NovaSeq platform providing 250bp paired-end (PE) reads for 50,000 tags per sample per amplicon.

Metabarcoding

The amplicon 250PE sequences were filtered, denoised and merged to produce amplicon sequence variants (ASVs), using *DADA2* in the QIIME2 (version 2022.8) platform (Bolyen et al. 2019). Analysis of *rbc*L sequence used merged paired-end and the forward read only was used for *trn*L sequences. This was because the merging rate was low in *trn*L sequences (<10 % for most of the samples) compared to *rbc*L (>80 %). The *trn*L region is typically more than 500 bp long so 250 bp PE reads do not overlap, whereas the 379 bp *rbc*L fragment was easily covered by 250 bp sequences from either end of the fragments. After filtering and denoising (and merging in case of *rbc*L), ASV sequences were blasted against the global QIIME2 *rbc*L reference database (Dubois et al. 2022) or global custom QIIME2 *trn*L reference database. The custom reference database for *trn*L was created using the DB4Q2 workflow (Dubois et al. 2022), after retrieving FASTA nucleotide data from the NCBI website (https://www.ncbi.nlm.nih.gov/) accessed on 8th October 2022 using the following *Entrez* text query: ("Viridiplantae"[Organism] AND (trnL[Gene Name] OR tRNA-Leu[Title] OR (trnL-trnF[Title] AND intergenic spacer[Title]) OR trnL[Title])

The presence/absence of identified plant taxa in Mount Hutt were confirmed using New Zealand Plant Conservation Network database (https://www.nzpcn.org.nz/) and iNaturalist (https://www.inaturalist.org/). Details of commands used in QIIME2 are provided in Supplementary Material.

3.3 Results

Mandible analysis

Morphometric data were obtained from mandibles of 124 grasshoppers (Supplementary Table 3.2). Principal component analysis (PCA) of mandible size and shape revealed that male and female *S. australis* and *P. nitidus* have distinctive shape features, in contrast to the overlap in PC distribution scores from female and male *B. nivalis*. The largest component (PC1) explained 75.8 % of variation contributed by length of incisor, molar and articulation hinge, suggesting this component is largely describing size variation among the grasshopper mandibles. The second largest component of variation (PC2; 20.4 %) partially separated individuals according to species (Figure 3.7) and reflected differences in the area of mandibular melanization.

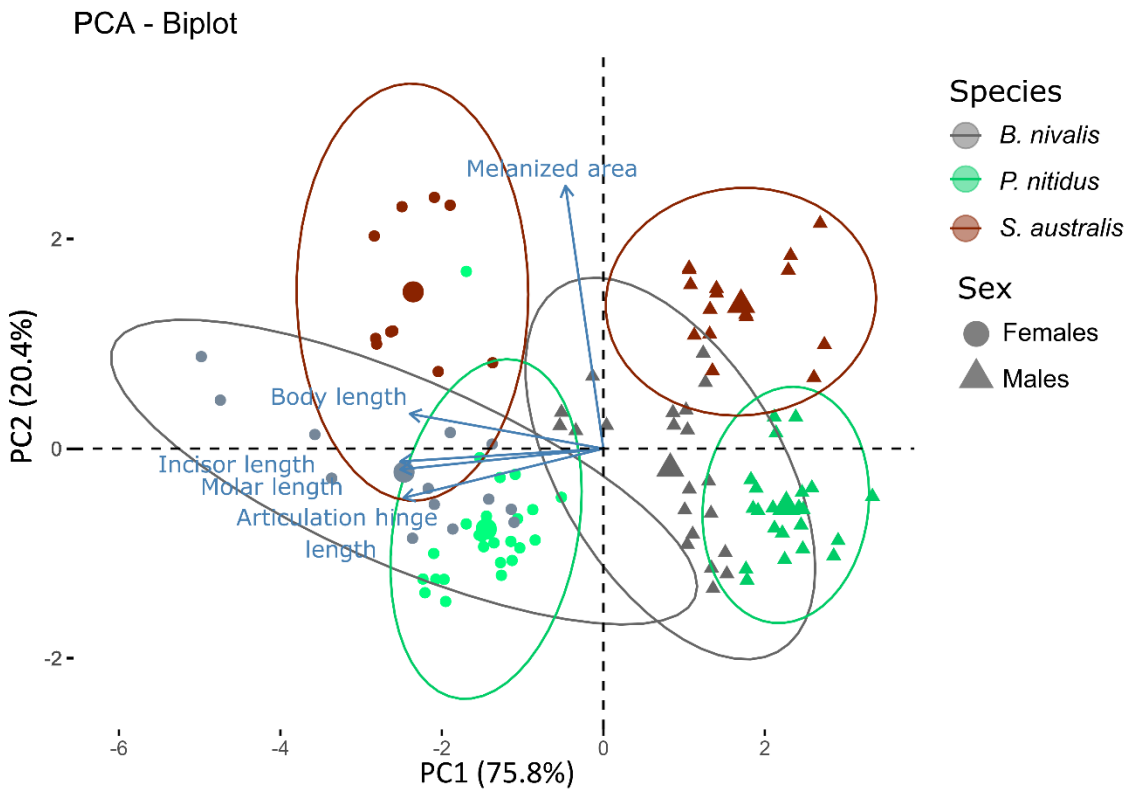

Figure 3.7 Principal component analysis (PCA) of mandibular traits (incisor length, molar length, articulation hinge length and melanized area) and body lengths of New Zealand grasshopper *Sigaus australis*, *Paprides nitidus* and *Brachaspis nivalis*. The ellipses show the 95% confidence areas around the centroids for each species and sex.

The three alpine grasshopper species showed sexual size dimorphism, with females having larger mandibles and bodies than their respective males (Figure 3.8A–C, E). In contrast, no sexual difference was observed in the proportion of their mandibles that was melanized. Male *B. nivalis* had significantly larger mandibles than males of other species (Figure 3.8A–C), but their body size was equal to *S. australis* (Figure 3.8E). Both males and females of *S. australis* have the largest area of melanization (mean±SD = 80.06±16.02 % in females and 69.75±9.48% in males), followed by *B. nivalis* (44.62 ±13.54 % in females, 37.55±16.68 % in males) and then *P. nitidus* (28.43±13.74 % in females, 26.01±9.99 % in males) (Figure 3.8D).

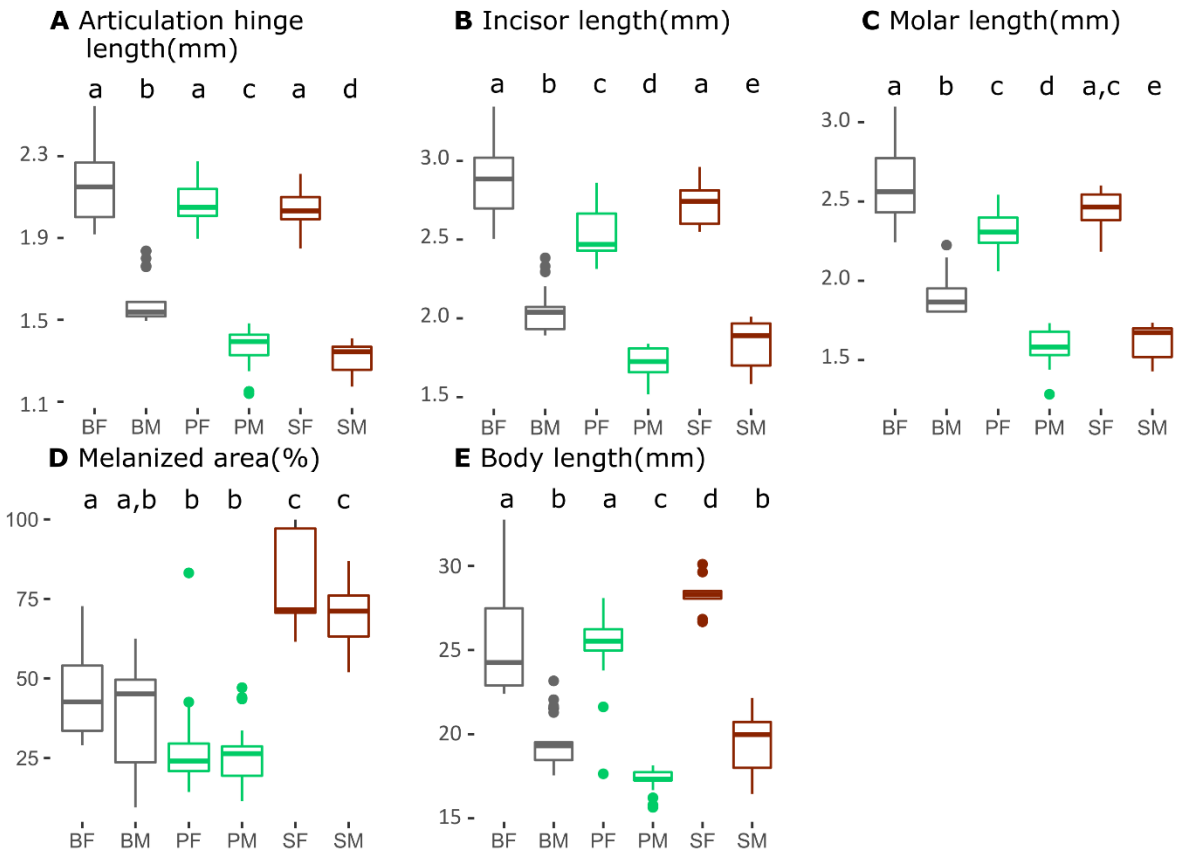

Figure 3.8 Variation in mandible traits (**A–D**) and body length (**E**) in three New Zealand alpine grasshopper species. Vertical bars indicate standard error. Different letters indicate significant differences between males and females within a species. Differences across species revealed from a linear model followed by a pair-wise post hoc Tukey honest significant test. Abbreviations: BF, *Brachaspis nivalis* female; BM, *B. nivalis* male; PF, *Paprides nitidus* female; PM, *P. nitidus* male; SF, *Sigaus australis* female; SM, *S. australis* male Different letters indicate significant differences between males and females within a species.

Plant epidermis analysis

In the contents of mid and hind guts of grasshoppers from Mount Hutt, plant fragments were present with the characteristic stomatal and epidermal cells of *Gentianella corymbifera, Celmisia discolor, Anisotome aromatica, Luzula rufa, Epilobium* species and *Gaultheria* species (Table 3.1; Figure 3.9). *Gaultheria* and *Epilobium* could not be identified to species due to morphological similarity of epidermal tissues (e.g., between *G. depressa* and *G. crassa*: Figure 3.6R & T). Some epidermal cells were not identified as they did not match any of the 31 reference plant species or as their characteristic structures were obscured (e.g.,

Figure 3.9M). Hair-like structures (Figure 3.9O) found in mid- and hind- gut made it difficult to dissociate plant parts resulting in low resolution of some plant fragments. These hair-like structures are not trichomes of plants as trichomes are much thicker (5–15µm in hair-like structures; typically 50–100µm in trichomes e.g., *Celmisia* species, *Hypochaeris radicata*, *Hieracium officinarum*). Gut contents of all grasshopper samples contained xylem or phloem tissues (Figure 3.9P) which could not be identified to plant species or types (monocots or dicots).

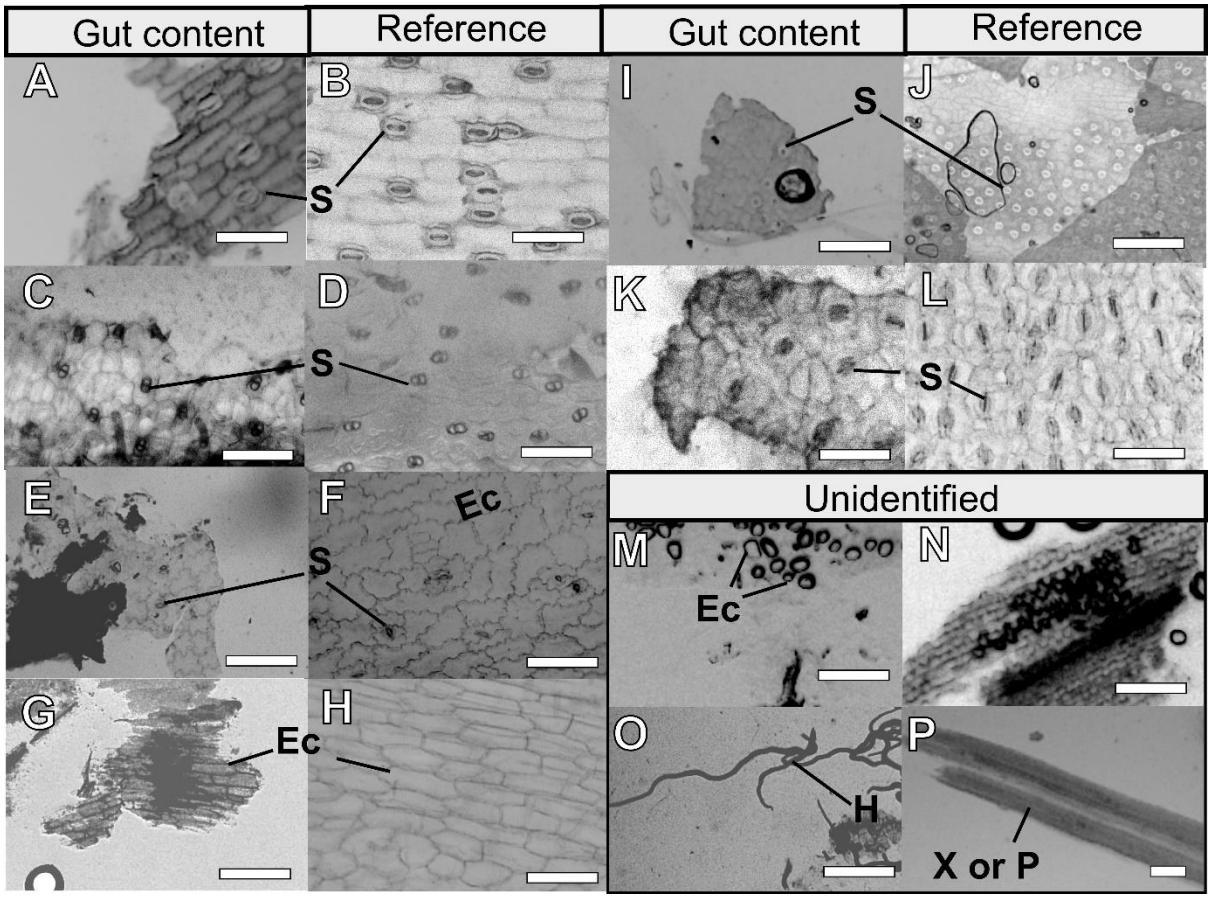

Figure 3.9 Plant tissue fragments from the contents of the mid- and hind-guts of New Zealand alpine grasshoppers, observed under light microscopy. *Paprides nitidus* gut content (**A**) and *Luzula rufa* (**B**); *Brachaspis nivalis* gut content (**C**) and *Celmisia discolor* (**D**); *P. nitidus* gut content (**E**) and *Gentianella corymbifera* (**F**); *B. nivalis* female gut content (**G**) and *Epilobium* species (**H**); *P. nitidus* male gut content (**I**) and *Anisotome aromatica* (**J**); *P. nitidus* male gut content (**K**) and *Gaultheria depressa* (**L**); unidentified epidermal cells covered with air bubbles (**M**); unidentified plant parts **(N**); hair-like structures found in mid- and hind-guts (**O**); xylem or phloem (**P**). Scale bars = 200 µm. Abbreviations: S = stoma(ta), Ec = epidermal cell, X = xylem, P= phloem.

Table 3.1 Plants identified from tissue fragments in the mid and hind guts of New Zealand alpine grasshoppers *Brachaspis nivalis*, *Paprides nitidus* and *Sigaus australis* in the Mount Hutt area.

Family	Species	*B. nivalis*				*P. nitidus*				*S. australis*			
		BF1	BF2	BM1	BM2	PF1	PF2	PM1	PM2	SF1	SF2	SM1	SM2
Juncaceae	*Luzula rufa* (Figure 9A, B)		+	+	+		+		+				
Asteraceae	*Celmisia discolor* (Figure 9C, D)	+											
Gentianaceae	*Gentianella corymbifera* (Figure 9E, F)						+		+	+			
Onagraceae	*Epilobium* spp. (Figure 9G, H)		+										
Apiaceae	*Anisotom aromatica* (Figure 9I, J)		+						+		+	+	+
Ericaceae	*Gaultheria* spp. (Figure 9K, L)							+					

DNA metabarcoding

A total of 1422 amplicon sequence variants (ASVs) of *rbc*L, and 9021 ASVs of *trn*L were initially identified from crops of 12 grasshoppers after filtering, denoising, merging and removing chimera (Table 3.2). Rare sequences that might be the result of sequencing error were discarded: ASVs containing <50 reads (*rbc*L) or <500 reads (*trn*L) were removed from the analysis (Table 3.3). This reduced the number of ASVs to 138 for *rbc*L, and 85 for *trn*L, while retaining >99% of reads for all samples.

Table 3.2 Plant chloroplast *rbc*L and *trn*L sequence reads from PCR amplicons of gut contents of 12 New Zealand alpine grasshoppers collected at Mount Hutt after filtering, denoising, merging (for *rbc*L) and removing chimeric sequences.

Species	Sex	Sample ID	Total sequences	Filtered	Denoised	Merged	Chimera removed
***rbc*L**							
B. nivalis	F	BF1	121659	107782	107613	107018	101019
		BF2	111538	98149	97848	97173	91994
	M	BM1	114520	100083	99437	93596	89627
		BM2	129647	113916	113409	112359	107984
P. nitidus	F	PF1	132535	115912	115368	112833	108899
		PF2	136575	112917	112357	94280	91938
	M	PM1	116057	101512	101297	100682	93457
		PM2	136358	119397	119105	118436	111997
S. australis	F	SF1	111371	98703	98544	98138	92372
		SF2	133630	117499	116887	115376	105046
	M	SM1	128495	112542	112393	111404	100375
		SM2	124271	109785	109497	108626	103223
***trn*L**							
B. nivalis	F	BF1	271040	248066	247120	NA	233537
		BF2	263601	232716	230770		221389
	M	BM1	120809	106943	102233		93953
		BM2	255380	228585	224327		218692
P. nitidus	F	PF1	251844	233207	231042		227800
		PF2	268206	242889	238745		224529
	M	PM1	282425	240277	239283		237275
		PM2	274978	233936	233382		228211
S. australis	F	SF1	265134	244851	243720		240002
		SF2	280379	253441	250755		241903
	M	SM1	250361	220117	219263		213791
		SM2	268475	248014	246531		241017

Table 3.3 Number of amplicon sequence variants (ASVs) before and after removal of rare ASVs (ASVs containing <50 reads in *rbc*L and <500 reads in *trn*L).

Species	Sex	Sample ID	*rbc*L		*trn*L	
			before	after	before	after
B. nivalis	**F**	BF1	55	25	228	30
		BF2	47	29	410	33
	M	BM1	321	67	1322	43
		BM2	170	26	1122	47
P. nitidus	**F**	PF1	286	20	604	49
		PF2	480	62	1423	37
	M	PM1	39	30	174	30
		PM2	28	22	80	28
S. australis	**F**	SF1	38	15	129	29
		SF2	174	29	849	30
	M	SM1	33	25	289	36
		SM2	61	20	287	28

The proportion of ASVs identified to family, genus and species level varied among samples (Supplementary Table 3.4). A larger proportion of *rbc*L ASVs were identified only to family level (on average 95.8%) than *trn*L ASVs (88.4%). The proportion of ASVs identified to genus was lower in *rbc*L ASVs (50.6%) than *trn*L ASVs (85.1%). Sequences identified to species were low for both genes (0.1% to species in *rbc*L and 0.5% in *trn*L), and plant species identified were *Calluna vulgaris* (Ericaceae) in *rbc*L and *Lobelia angulata* (Campanulaceae), *Chionochloa macra* (Poaceae), *Rumex acetosa* (Polygonaceae), and *Polytrichum juniperinum* (Polytrichaceae) in *trn*L. Due to low taxonomic resolution at species level, further results will be focused on family and genus levels.

For both genes, at least four plant genera were identified in each grasshopper crop sample (Figure 3.10). In most grasshopper crop samples, more than 60% of sequences were assigned to one of these four taxa: shrub *Gaultheria* (Ericaceae), rush *Luzula* (Juncaceae), dicot herb *Hieracium,* shrub *Olearia* (Asteraceae). The exceptions were one *B. nivalis* male (BM1) with 27.8% of *trn*L and 52.6% of *rbc*L sequences assigned to the shrub *Veronica* (Plantaginaceae) and one *S. australis* female (SF2) had 36.1% of *trn*L and 59.5% of *rbc*L sequences assigned to an unidentified Apiaceae taxon (Figure 3.10). The grass genus *Poa* (Poaceae) comprised <1% of sequences for all samples (both *rbc*L and *trn*L) except in one *S. australis* male (SM2) where 6.4% of *rbc*L and 1.2% of *trn*L sequences were assigned to *Poa* (Figure 3.10). The dicot family Gesneriaceae comprised <1% of sequences for all samples except one *B. nivalis* female (BF2) with 22.6% of *trn*L sequences assigned to Gesneriaceae. In all samples < 0.1% of *trn*L sequences were identified as *Chionochloa* (Poaceae) (not detected in *rbc*L) and <1% of *trn*L and *rbc*L sequences assigned to *Lobelia* (Campanulaceae), *Celmisia* (Asteraceae), *Lupinus* (Fabaceae), *Kelleria* (Thymelaeaceae). The dicot herb *Rumex* (Polygonaceae), *Epilobium* (Onagraceae), and *Gentianella* (Gentianaceae) comprised >1% of *trn*L sequences in some samples (Figure 3.10B). None of the plant families, genera or species identified were eaten by just one particular species or sex of grasshopper.

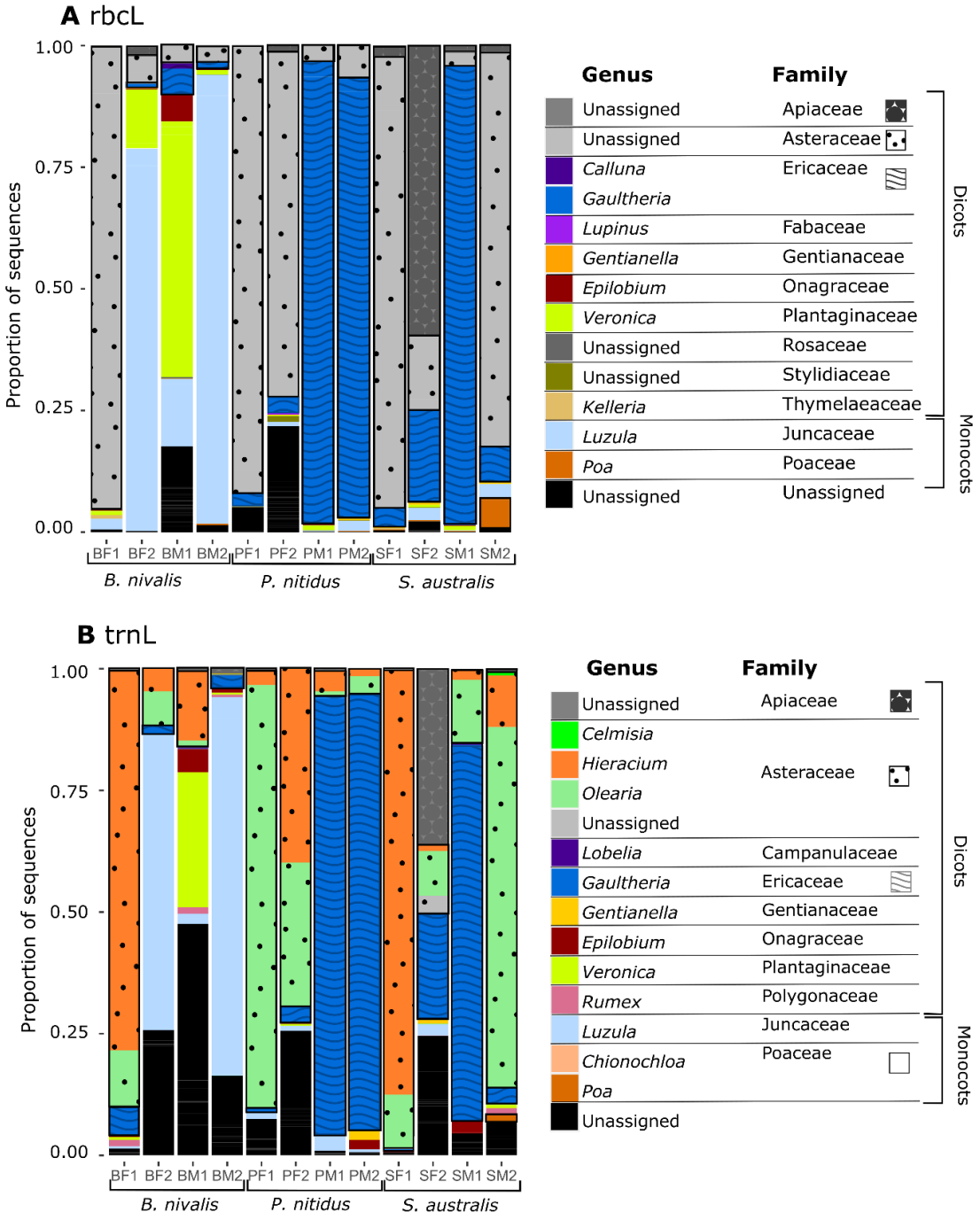

Figure 3.10 Proportion of *rbc*L (**A**) and *trn*L (**B**) sequences assigned to specific genus and family. Different genera represented in different colors and different families represented in different patterns (if multiple genera are included within a family). Abbreviations: BF = *Brachaspis nivalis* female, BM = *B. nivalis* male, PF = *Paprides nitidus* female, PM = *P. nitidus* male, SF = *S. australis* female, SM = *S. australis* male.

3.4 Discussion

Sympatric species are expected to respond to natural selection so that competition for the same resources is minimised, resulting in ecological differentiation (Kaplan and Denno 2007). However, biotic and indirect interactions rather than resources might limit abundance so that different species co-occur at the same location. To investigate potential diet differences among three sympatric grasshopper species, mandible morphology and gut contents were compared.

Mandible analysis

Insect mandibles reveal adaptation to their food plants (Clissold 2007; Krenn 2019). The three alpine grasshoppers studied here (*B. nivalis, P. nitidus* and *S. australis*) have mandibles that are similar to other dicot-feeding species (Figure 3.1; Isely 1944; Chapman 1964; Gangwere 1965; Kang et al. 1999). Mandible measurements (articulation hinge, incisor and molar lengths) showed that females have larger mandibles than males. Sexual dimorphism in mandible size was more apparent than variation among the three species suggesting that sex differences in diet may be larger than species differences. These results suggest that females may feed on plant species with thicker leaves than males.

The proportion of melanized area on the mandibles did not differ between sexes but *S. australis* had a larger melanized area than the other two species. As melanization in insects is related to cuticle hardening (Krenn 2019), it is possible that *S. australis* can feed on plants with tougher tissues compared to the other species. Toughness is a physical barrier against herbivores in plants and in one study, supplementation of the food grass *Bromus catharticus* with silicon increased the toughness of the plant and significantly reduced herbivory by the grasshopper *Oxya grandis* (Mir et al. 2019). Measuring toughness and size of grasshopper

food plants are further required to elucidate species and sex specific adaptation to food plants in New Zealand alpine grasshoppers.

Gut content analysis

Histology compared to DNA

Food plants were identified in males and females of three alpine grasshopper species using microhistological epidermal analysis of gut contents and by amplifying *rbc*L and *trn*L chloroplast genes of crop contents. Plant identification of grasshoppers with microhistological analysis has been used for 50+ years (Watson 1970; Joern 1979b) but the use of DNA sequences to identify the plant species eaten by herbivores has rapidly increased over the last twenty years (Avanesyan et al. 2021). Comparison of DNA to microhistological analysis shows an improved taxonomic resolution of plant identification (Soininen et al. 2009). In this study, I used the contents of the insect crop for DNA analysis and the contents of the mid and hind guts of the same specimens for histology. As DNA is expected to break down faster than cuticular structures, the plant fragments in the first stage of the digestive tract (crop) were considered to be optimal for amplicon sequencing. Many of the plant fragments were unidentified due to lack of distinguishing features and lack of reference material.

Sequences assigned to one of four genera (*Gaultheria* (Ericaceae), *Luzula* (Juncaceae), *Hieracium* and *Olearia* (Asteraceae)) were detected in high proportion (>60%) in 11 of the 12 grasshoppers sampled. *Gaultheria* and *Luzula* were detected in both DNA sequences and microhistological analysis of mid and hindgut contents. Unassigned Apiaceae DNA

sequences identified in crops are possibly *Anisotome aromatica*, which was detected in the mid- and hind-gut contents from epidermal structures.

The plants identified from crops and guts of the same individual were not always the same. For example, *Epilobium* was detected only from one *B. nivalis* female (BF1) in mid and hind guts but some of the *trn*L sequences were assigned to *Epilobium* in two *B. nivalis* males, one *P. nitidus* male (PM2) and one *S. australis* male (SM1). Although both *trn*L and *rbc*L DNA sequences were assigned to *Gaultheria* in 11/12 grasshoppers, *Gaultheria* was detected only from *P. nitidus* male (PM1) in microhistological analysis. This likely reflects difference in sensitivity of the two methods and different feeding events. A crop is a temporary food reservoir in insects before absorption and digestion process in mid and hindguts (Terra and Ferreira 2009) and these results may show that there is a time gap before the plant parts are passed from crop to mid-gut.

Comparing cpDNA regions

DNA metabarcoding allows identification to family, genus and sometimes to species level even with degraded plants (Soininen et al. 2009; McClenaghan et al. 2015; Mallott et al. 2018; Welti et al. 2019) as well as phloem and xylem that cannot be used for plant identification in microhistological analysis. On average, DNA metabarcoding allowed identification to plant families for 95.8% of *rbc*L and 88.4% of *trn*L sequences and even to genus (50.6% in *rbc*L and 85.1% in *trn*L) and species (0.1% in *rbc*L and 0.5% in *trn*L) in this study. The lower level of identification at family level for *trn*L DNA sequences possibly relates to the length of the DNA fragments (shorter for *trn*L as only one of the two paired end reads was used in this analysis), or to reference sequence data availability (Mallott et al. 2018). Better taxonomic resolution in *trn*L than *rbc*L is because the DNA sequence of the

*trn*L-c and *trn*L-d region is more variable as it includes a group I intron which is non-coding (Taberlet et al. 2007) and therefore more taxon-specific than *rbc*L. Overall, DNA metabarcoding using *trn*L would be better for taxonomic identification in plants.

One study showed taxonomic identification using global sequence database allowed only genus or family identification even in high-resolution *trn*L genes while using local database allowed identification to species level (Nakahara et al. 2016). In this study, low resolution of species identification using global database was also observed for both *rbc*L and *trn*L sequences. Therefore, preparing local-based database may improve the taxonomic resolution, although creating reference database DNA metabarcoding for all available plants is time-consuming and expensive.

Comparing plants in gut with plants in habitat

Sequences assigned to *Gaultheria* (Ericaceae) or *Luzula* (Juncaceae), were detected in high proportion (>60 %) in five of the twelve grasshoppers I sampled from Mount Hutt. As these two plant genera were rare components of the vegetation but found in relatively high frequency in grasshopper gut contents, Watson (1970) considered them to be highly preferred food plants for all three alpine grasshopper species (Supplementary Table 3.1). In contrast, the tussock *Chionochloa* is the dominant species in Craigieburn (comprising >30% vegetation) but was rare in the grasshopper gut contents and therefore considered to be the least favoured plant by alpine grasshoppers (Watson 1970). I found *Chionochloa* comprised less than 0.1% of *trn*L sequences and was not detected in *rbc*L sequences or microhistological analysis, despite its high abundance at Mount Hutt where the grasshoppers were caught. Another common DNA sequence in the gut of the Mount Hutt grasshoppers was the hawkweed *Hieracium* species which was rare 50 years ago and the only species recorded

in Watson's observation in Craigieburn Range (1970) was *H. argillaceum. Hieracium* are invasive weeds in the New Zealand high country and have increased their distribution and abundance in the past 50 years (Meffin 2010; Steer and Norton 2013; Jensen et al. 2019) and thus possibly became an important component of alpine grasshoppers' diet.

Comparing sex and species

By sampling three grasshopper species from the same location (within a few meters of one another) on the same day, I reduced the potential confounding effects of season and location on diet. Despite the differences in mandible size and melanization, no species- or sex-specific plant species were detected. Only *B. nivalis* specimens had high abundance of sequences from the rush *Luzula* spp. and the shrub *Veronica* species but larger sample sizes would be needed to infer anything from this observation. It is possible that the morphological differences in their mandibles allow grasshoppers to feed on different plant tissues rather than different plant species.

Combining histology and metabarcoding has the advantage that a full list of plant species can be determined as well as the ability to identify and compare different plant tissue types (seeds, leaves, stalks, flowers). Resource-partitioning might exist at the level of macronutrient use rather than discrete plant taxa (Behmer and Joern 2008) with differences in the proportion of species and tissues eaten. It has been estimated that 10 –18 % of the diet of these alpine grasshoppers is made up of flowers (Watson 1970). Seasonal observations showed that ingestion frequency of flowers increased during the flowering season (December to February: Watson 1970) suggesting flowers are highly preferred by these alpine grasshoppers. However, examination of plant fragments has the disadvantage that plant tissues do not digest at a constant rate and many structures such as flowers and seeds are

likely to be unidentifiable. These plant tissues do of course contain DNA and therefore there is the potential for amplicon DNA sequencing and metabarcoding to provide an improved snapshot of the insects' diet. Although DNA metabarcoding does not allow identification of plant tissues (e.g., leaf vs. flower) eaten, it is possible that some sequences are derived from flower parts. If grasshoppers were eating the reproductive structures of particular plant species they might have a significant impact. Knowing what a grasshopper eats could be useful for understanding their potential ecological impact, in the case of pest species (e.g., locusts: Cumberland et al. 2017), and for revealing their influence in shaping native plant communities and shedding light on competitive interactions. Despite the small sample size used here, this study demonstrates that the amplicon metabarcoding approach has advantages for identifying biologically important elements of the diet of herbivores and findings here support the idea that these grasshopper species have important selective role determining the success of plant species in the alpine zone.

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3.6 Supplementary Materials

Below is the command used on QIIME2 for bioinformatic analysis:

1. Converting data

#fastq -> qza format (QIIME2 format)

Ref: https://docs.qiime2.org/2022.8/tutorials/importing/?highlight=tool%20import

```
qiime tools import \
--type 'SampleData[SequencesWithQuality]' \
--input-path manifest.txt \
--output-path single-end-demux.qza  \ *paired-end-demux.qza if paired-end sequences
--input-format SingleEndFastqManifestPhred33V2
```

2. *DADA2* denoising

#Denoising

Ref: https://docs.qiime2.org/2022.8/plugins/available/dada2/denoise-single/

```
qiime dada2 denoise-single \ *qiime dada2 denoise-paired if paired-end sequences
--i-demultiplexed-seqs single-end-demux.qza \
--p-trunc-len-f 0 \
--p-trunc-len-r 0 \
--o-table table.qza \
--o-representative-sequences rep-seqs.qza \
--o-denoising-stats denoising-stats.qza
```

3. Data visualization (qzv files can be visualized in https://view.qiime2.org/)

#Denoising summary: percentage and number of sequences after filtered, denoised, chimera removal (and merged if paired-end)

```
qiime metadata tabulate \
--m-input-file denoising-stats.qza \
--o-visualization denoising_stats
```

#ASV summary table: list of consensus sequences and sequence length statistics

qiime feature-table tabulate-seqs \

--i-data rep-seqs.qza \

--o-visualization rep-seqs

#ASV table: visualize the number of sequence per sample per ASV

qiime tools export

--input-path table.qza

--output-path table

4. Blasting ASVs against NCBI custom reference database

qiime feature-classifier classify-consensus-blast \

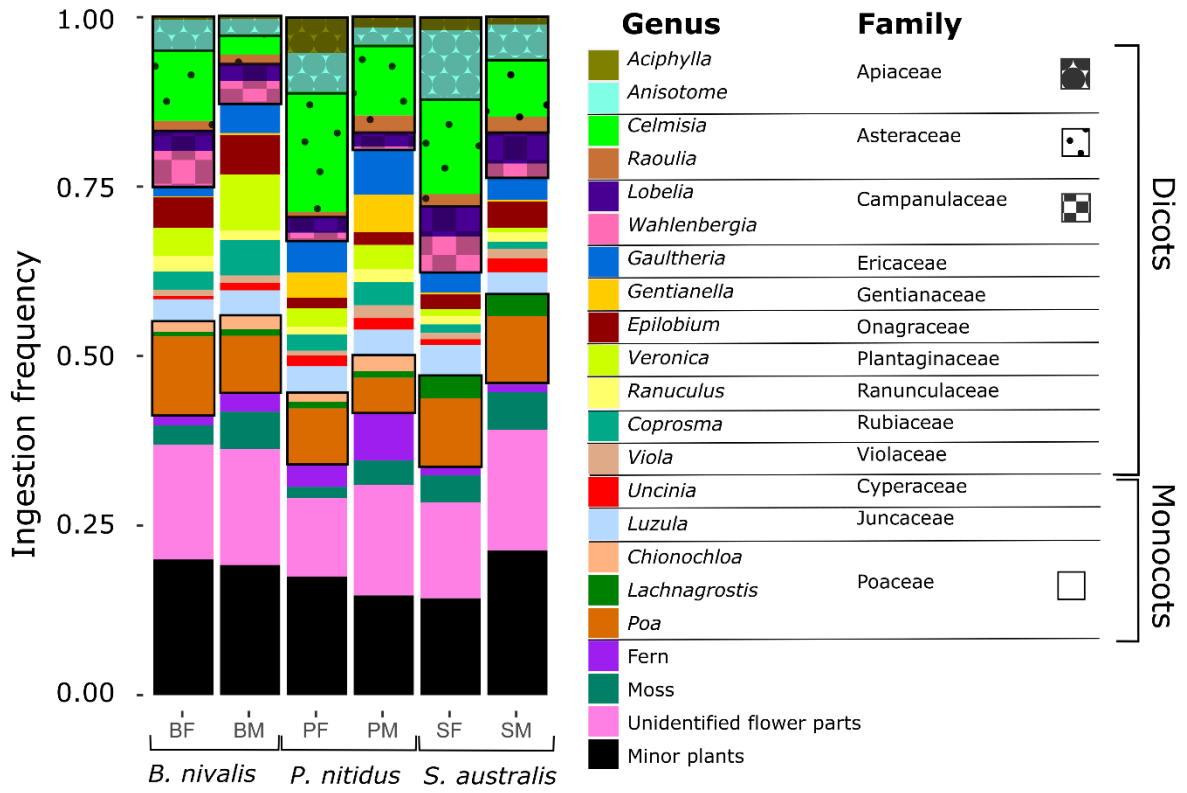
--i-query rep-seqs.qza \

--i-reference-reads NCBI_fasta_file.qza \ *created using Dubois et al. 2022

--i-reference-taxonomy NCBI_taxonomic_lineages.qza \ *created using Dubois et al. 2022

--o-classification classified_sequence.qza \

--o-search-results searchresults.qza



Supplementary Figure 3.1 Average ingestion frequency of plant genera and families observed in males and females of New Zealand alpine grasshopper *Brachaspis nivalis*, *Paprides nitidus* and *Sigaus australis* (data derived from Watson 1970). Ingestion frequency was calculated by dividing the number of ingestion recordings of particular plant by total number of plant ingestion recordings. Different genera represented in different colors and different families represented in different patterns (if multiple genera are included within a family). Abbreviations: BF = *Brachaspis nivalis* female, BM = *B. nivalis* male, PF = *Paprides nitidus* female, PM = *P. nitidus* male, SF = *S. australis* female, SM = *S. australis* male.

Supplementary Table 3.1 Preference level of plant species occurred in high frequency of gut contents of New Zealand alpine grasshoppers. Preference level measured by comparing ingestion frequency to vegetation frequency (derived from Watson 1970). Taxonomic name in bracket is the previously used name.

Preference level	Family	Species	Structural type
Low	Poaceae	*Chionochloa* species	grasses
	Poaceae	*Rytidosperma setifolium* (*Notodanthonia setifolia*)	grasses
	Poaceae	*Festuca novae-zelandiae*	grasses
	Asteraceae	*Celmisia lyallii*	dicot herbs
	Ericaceae	*Dracophyllum pronum*	shrubs
Medium	Poaceae	*Poa colensoi*	grasses
	Asteraceae	*Celmisia spectabilis* *Celmisia viscosa*	dicot herbs
	Apiaceae	*Aciphylla monroi*	dicot herbs
	Blechnaceae	*Blechnum penna-marina*	ferns
	Lycopodiaceae	*Lycopodium fastigiatum*	ferns
	Polytrichaceae	*Polytrichum juniperinum*	mosses
High	Juncaceae	*Luzula rufa*	rushes
	Apiaceae	*Anisotome aromatica*	dicot herbs
	Campanulaceae	*Lobelia angulata* (*Pratia angulata*) *Wahlenbergia albomarginata*	dicot herbs
	Ericaceae	*Gaultheria depressa*	shrubs

Supplementary Table 3.2 Collected sites (BR = Broken River Ski Area (-43.125750, 171.686239), MH = Mount Hutt Ski Area (-43.5118, 171.5492 for mandible analysis; -43.495870, 171.539220 for gut content analysis), FP = Fox Peak Ski Area (-43.8530, 170.8077)) and year and sample size of New Zealand alpine grasshoppers used in mandible analysis and DNA analysis.

	Species	Sex	Population	Collection Year	Sample Size
Mandible analysis	*Brachaspis nivalis*	F	BR	2021	10
			MH	2016	9
			FP	2016	1
		M	BR	2021	11
			MH	2016	8
			FP	2016	5
	Paprides nitidus	F	BR	2021	7
			MH	2016	13
			FP	2016	2
		M	BR	2021	12
			MH	2016	9
			FP	2016	3
	Sigaus australis	F	BR	2021	10
			MH	2016	4
			FP	2016	4
		M	BR	2021	6
			MH	2016	5
			FP	2016	5
Gut content analysis	*Brachaspis nivalis*	F	MH	2022	2
		M	MH	2022	2
	Paprides nitidus	F	MH	2022	2
		M	MH	2022	2
	Sigaus australis	F	MH	2022	2
		M	MH	2022	2

Supplementary Table 3.3 List of plant species collected for the reference materials of microhistological epidermal analysis. Specimens were collected from Broken River Ski Area (-43.125750, 171.686239) in 2021 and Tongariro National Park (-39.197260,175.564351) and Foggy Peak (-43.294107,171.744770) in 2022.

Population	Structural type		Family	Species
Broken River Ski Area	Monocot	Grass	Poaceae	*Chionochloa macra*
			Poaceae	*Chionochloa pallens*
			Poaceae	*Poa colensoi*
			Poaceae	*Rytidosperma*
	Dicot	Herb	Apiaceae	*Aciphylla aurea*
			Apiaceae	*Aciphylla monroi*
			Apiaceae	*Anisotome aromatica*
			Asteraceae	*Celmisia discolor*
			Asteraceae	*Celmisia lyallii*
			Asteraceae	*Celmisia spectabilis*
			Asteraceae	*Celmisia viscosa*
			Onagraceae	*Epilobium perplexum*
			Gentianaceae	*Gentianella corymbifera*
			Polygonaceae	*Rumex acetosella*
			Campanulaceae	*Wahlenbergia albomarginata*
	Dicot	Shrub	Ericaceae	*Dracophyllum* spp.
			Ericaceae	*Gaultheria depressa*
			Podocarpaceae	*Podocarpus nivalis*
			Plantaginaceae	*Veronica epacridea*
			Plantaginaceae	*Veronica lycopodioides*
			Plantaginaceae	*Veronica odora*
			Plantaginaceae	*Veronica pinguifolia*
	Fern		Blechnaceae	*Blechnum penna-marina*
			Blechnaceae	*Blechnum procerum*
Foggy Peak	Monocot	Grass	Poaceae	*Chionochloa* spp.
		Rush	Juncaceae	*Luzula rufa*
	Dicot	Herb	Asteraceae	*Celmisia spectabilis*
			Asteraceae	*Hypochaeris radicata*
			Asteraceae	*Pilosella officinarum*
			Gentianaceae	*Gentianella corymbifera*
			Gentianaceae	*Gentianella bellidifolia*
			Campanulaceae	*Wahlenbergia albomarginata*
	Dicot	Shrub	Coriariaceae	*Coriaria arborea*
			Ericaceae	*Gaultheria crassa*
Tongariro National Park	Monocot	Rush	Juncaceae	*Luzula rufa*
	Dicot	Herb	Asteraceae	*Pilosella officinarum*
		Shrub	Ericaceae	*Gaultheria antipoda*

Supplementary Table 3.4 Comparison of plant taxonomic identification (%) from *rbc*L and *trn*L chloroplast to family, genus and species level. Shading indicates a higher taxonomic identification from *rbc*L or *trn*L of the same sample.

	***rbc*L**				***trn*L**			
	Brachaspis nivalis							
	BF1	**BF2**	**BM1**	**BM2**	**BF1**	**BF2**	**BM1**	**BM2**
Order	99.4%	99.8%	82.3%	98.6%	99.0%	75.2%	62.5%	86.9%
Family	99.4%	99.8%	82.3%	98.6%	99.0%	75.1%	62.5%	86.9%
Genus	4.0%	91.9%	78.7%	95.1%	98.9%	75.1%	62.1%	85.9%
Species	0.0%	0.0%	1.1%	0.0%	1.3%	0.0%	1.8%	0.7%
	Paprides nitidus							
	PF1	**PF2**	**PM1**	**PM2**	**PF1**	**PF2**	**PM1**	**PM2**
Order	94.9%	78.2%	99.8%	99.9%	91.7%	79.3%	99.2%	99.5%
Family	94.9%	78.2%	99.8%	99.9%	91.7%	79.3%	99.2%	99.5%
Genus	2.7%	4.9%	96.6%	93.1%	91.6%	79.2%	98.8%	99.5%
Species	0.0%	0.0%	0.0%	0.0%	0.2%	0.0%	0.0%	0.2%
	Sigaus australis							
	SF1	**SF2**	**SM1**	**SM2**	**SF1**	**SF2**	**SM1**	**SM2**
Order	99.6%	97.8%	99.9%	99.3%	99.5%	77.7%	96.5%	94.3%
Family	99.6%	97.8%	99.9%	99.3%	99.5%	77.7%	96.5%	94.3%
Genus	4.5%	22.6%	95.8%	17.1%	99.5%	40.5%	96.1%	93.5%
Species	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.3%	1.5%

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Chapter 4

Abundance and distribution of antennal sensilla on males and females of three sympatric species of alpine grasshopper (Orthoptera: Acrididae: Catantopinae) in Aotearoa New Zealand

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Abundance and distribution of antennal sensilla on males and females of three sympatric species of alpine grasshopper (Orthoptera: Acrididae: Catantopinae) in Aotearoa New Zealand

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Abstract

Brachaspis nivalis, *Sigaus australis* and *Paprides nitidus* are grasshopper species endemic to Aotearoa, New Zealand where they are sympatric in several regions of South Island. On mountains of Kā Tiritiri o te Moana (Southern Alps), *B. nivalis* is more abundant on scree/rock habitat, whereas *S. australis* and *P. nitidus* are prevalent in alpine tussock and herbfields. It is expected, therefore, that these species have different sensory needs that are likely to be apparent in the type, abundance, and distribution of chemo-sensilla on their antennae. It is also likely that natural selection has resulted in sexual differences in sensilla. To test these hypotheses, abundance and distribution of the chemo-sensilla on the dorsal and ventral surfaces of their antennae were characterized in adult males and females of the three species. Five types of chemo-sensilla were identified on the distal portion of their antenna: chaetica, basiconica, trichoidea, coeloconica, and cavity. All species had significantly more chemo-sensilla on the ventral than the dorsal surface of antennae and a similar distribution pattern of chemo-sensilla. Despite having relatively short antenna, *B. nivalis* had the largest number of olfactory sensilla, but the fewest chaetica of the three species studied. A plausible explanation is that *B. nivalis* is abundant on less vegetated habitats compared to the other species, and therefore may rely more on olfaction (distance) than gustatory (contact) reception for finding food. No significant differences were observed between the sexes of *B. nivalis* and *P. nitidus*, however, *S. australis* males had significantly more basiconica sensilla than females.

Keywords Acrididae · Antenna · Sensilla · Sexual dimorphism · Sympatry

Introduction

A sensillum is a sensory organ protruding through the impervious exoskeleton of an insect, allowing detection of chemicals, temperature, and movement (e.g., olfactory, gustatory, mechanical, hygro-receptive and thermo-receptive sensilla). In short-horned grasshoppers (Orthoptera, Acrididae), chemical sensitive sensilla are abundant on structures including antennae (Altner et al. 1981; Bland 1989; Chapman 1989; Chen et al. 2003; Greenwood and Chapman 1984; Li et al. 2007; Ochieng et al. 1998; Roh et al. 2020), mouthparts (Blaney and Chapman 1969; Chapman 1989; Jin et al. 2006), legs (Mücke 1991; Yu et al. 2011) and wings (Zhou et al. 2008). The function of each sensilla can be inferred from its shape, size, presence and absence of pores and socket type (Bland 1989; Chapman 1989; Chen et al. 2003; Garza et al. 2021; Li et al. 2007; Nowińska and Brożek 2017). For example, sensilla without pores (aporous) and a flexible socket are considered to be mechano-receptors, whereas sensilla with pore(s) and an inflexible socket are considered to be chemical receptors (Garza et al. 2021; Li et al. 2007; Nowińska and Brożek 2017; Roh et al. 2020). Chemo-sensitive sensilla can have a single hole (uniporous) at the tip of the projection (apical pore) or have many pores (multi-porous or wall-pored), and these sensilla are responsible for gustation (contact chemoreception) and olfaction (distance chemoreception) respectively. The number and proportions of different types of sensilla are likely to be species-specific and comparison of sensilla density and morphology among species can reveal important ecological differences (Nakano et al. 2022).

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The abundance of sensilla of various types appears to be related to several ecological factors including the dietary range (i.e., monophagous, oligophagous, polyphagous: Bland 1989; Chen et al. 2003; Zaim et al. 2013), distribution and abundance of resources (i.e., mates and food: Greenwood and Chapman 1984; Ochieng et al. 1998) and sexual communication (i.e., signalers and receivers: Bland 1989; Chen et al. 2003; Li et al. 2007, 2021a; Malo et al. 2004; Roh et al. 2016). The inference that the sensitivity of an insect to its external environment depends on the abundance of sensilla (Bland 1989; Chapman 1989) is supported by observations using electro-physiological techniques, such as electroantennography (EAG) and single sensillum recordings (SSRs) (Ochieng and Hansson 1999; Chen and Kang 2000; Malo et al. 2004; Li et al. 2021a). For example, the different phases of locusts show characteristic abundance of sensilla on their antenna. Solitarious locusts (at low density) possess more olfactory sensilla (Ochieng et al. 1998) with higher electrophysiological responses to some pheromone components compared to their high density gregarious phase (Ochieng and Hansson 1999). This is possibly because the solitarious locusts require higher olfactory sensitivity to locate conspecifics under low population density compared to the gregarious phase (Hassanali et al. 2005). Sexual role is also linked to sensilla abundance and distribution, where receivers (typically males) have higher abundance of sensilla with higher olfactory sensitivity than signalers (typically females), as observed in a range of insects including grasshoppers (Chen and Kang 2000), beetles (Li et al. 2021a,b) and moths (Malo et al. 2004). A greater abundance of chemo-receptive sensilla is therefore predicted for those species that live in habitats with sparsely distributed resources and in the sex that is responsible for receiving chemical signals during mating (typically males).

The approximately 12,250 species of grasshoppers (Orthoptera; Caelifera) interact with diverse plant communities around the globe (Husemann et al. 2022; Ibanez et al. 2013; Joern 1979; Welti et al. 2019). However, most current knowledge of the chemical exchanges that underpin these plant–insect interactions is derived from the study of a small number of economically important pest species (locusts) (Nakano et al. 2022). In addition to locust species, representatives of a number of Gomphocerinae, Oedipodinae and Melanoplinae, and a few species from Acridinae (Bland 1982, 1989; Chen et al. 2003; Li et al. 2007) have been examined for sensilla but no representatives of the Euryphyminae, Eyprepocnemidinae, Ommatolampidinae, Spathosterninae, Coptacrinae, or southern Catantopinae.

The alpine environment of Aotearoa/New Zealand has a rich, endemic ecological community including flightless, acridid grasshoppers (Bigelow 1967; White 1975). These species of southern Catantopinae are the products of an endemic radiation associated primarily with Kā Tiritiri o te Moana, the Southern Alps (Koot et al. 2020). At most locations, several species co-occur on the same plant communities with overlap in their food plants (Watson 1970). Three widespread sympatric species, *Brachaspis nivalis* (Hutton, 1898), *Sigaus australis* (Hutton, 1897) and *Paprides nitidus* (Hutton, 1898), have been shown to have different micro-habitat preferences within scree-shrub-herbfield mosaics (Bigelow 1967; Koot 2018; Watson 1970). Habitat partitioning suggests that these grasshopper species have different sensory requirements relating to the type and distance of cues from potential food plants. Similarly, communication between individual grasshoppers exerts specific demands on sensory ability. The coloring and appearance of these grasshoppers suggests selection on camouflage from predators rather than sexual signals (Fig. 1), and they have reduced wings (tegmina) unsuitable for sound production. Together these limitations in auditory and visual signaling imply that chemical cues may be important for selection of mates as well as food, but direct evidence is lacking.

To explore the chemosensory capabilities of endemic, flightless grasshoppers, we use a comparative approach, hypothesizing that sensilla abundance and distribution among these three species will reflect the putative ecological differences of co-occurring taxa. We focused on antennal sensilla, as the antenna is the major location for chemical receptive sensilla (Bland 1989; Chen et al. 2003). We predicted more sensilla on the antennae of *B. nivalis* that is predominantly in rocky areas of sparse vegetation, compared to *S. australis* and *P. nitidus*. We also expected that sexual dimorphism in antennal chemosensory structures would be apparent with males (potential signal-receivers) having higher densities of sensilla than females (Bland 1989; Chen et al. 2003; Li et al. 2007). We quantified the abundance and distribution of chemo-sensilla in male and female *B. nivalis*, *S. australis* and *P. nitidus*.

Materials and methods

Insects

Adult grasshoppers of *B. nivalis*, *S. australis* and *P. nitidus* (Fig. 1) were collected during the active summer season on the southeast flank of Hamilton Peak in the Craigieburn Range (43′07′3″0.7″S 171′41′1″0.5″E) with approval from the Broken River ski area operators and New Zealand Department of Conservation (authorization number: 97397-FLO). Insect specimens were frozen then preserved in 99% ethanol. Storage in high concentration ethanol preserved DNA and effectively dehydrates tissues for microscopy.

Fig. 1 Three sympatric New Zealand alpine grasshoppers are cryptically colored in their typical habitat. *Brachaspis nivalis* adult male (**a**), *Sigaus australis* adult female (**b**), *Paprides nitidus* adult male (**c**), *B. nivalis* adult female (**d**), *S. australis* adult female (**e**), *P. nitidus* adult male (**f**)

Scanning electron microscopy (SEM)

Antennae were examined under a scanning electron microscope (SEM) after being excised from preserved specimens and fixed in fresh 99% ethanol for one to three days to ensure dehydration, and then air-dried for two days. Fixed antennae were mounted on aluminum stubs, and gold-coated for 200 s with a Baltec SCD 050 sputter coater before examination with an FEI Quanta 200 SEM operated in the range of 15–20 kV.

Antenna size and sensilla

Antennal morphology was examined under a Leica stereo microscope (SM225, Olympus, Japan) equipped with a digital camera (SC180, Olympus, Japan) and antennal lengths were measured using imaging software (NIS-Elements 5.01, Nikon Instruments Inc., USA), at The New Zealand Institute for Plant & Food Research Limited, Palmerston North, with permission from Dr Kambiz Esfandi. The area of each antennal segment was measured using the Measure function on ImageJ/Fiji with SEM images.

Dorsal and ventral surfaces of either a left or right antenna of each adult grasshoppers were examined for 10 or 11 males and 10 or 11 females of each species. The surface of each antenna was identified by its position in relation to the antennal groove on the frons (Fig. 2), with the presence of a lenticular organ on the ventral surface of segment 14 and the dorsal surface of segment 20 providing confirmation (Fig. 3a, b; Chen et al. 2003; Bland 1989). These grasshoppers have 23 segments on their antenna, but some individuals have subsections within particular segments (Fig. 3c, d), but we ensured consistent segment numbering by measuring the area of each segment (Table S1). The thirteen distal segments (segments 11 to 23; counting from scape, 23rd being the most distal) are those on which chemo-sensitive sensilla have been reported as abundant in other grasshopper species, whereas the proximal segments have sensilla usually linked to proprioception (Bland 1982, 1989; Chen et al. 2003; Jin

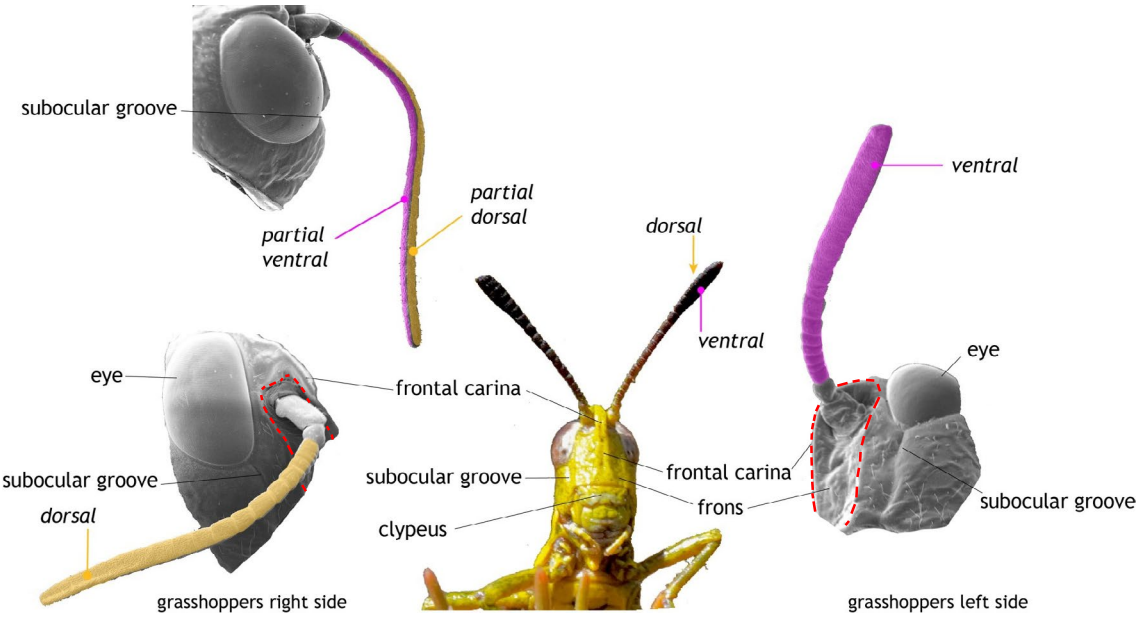

Fig. 2 Identification of ventral (purple) and dorsal (yellow) surfaces of antenna in New Zealand alpine grasshoppers. The surfaces of the antennae were determined by orientation relative to the groove (indicated by red dotted line) between frontal carina and subocular groove

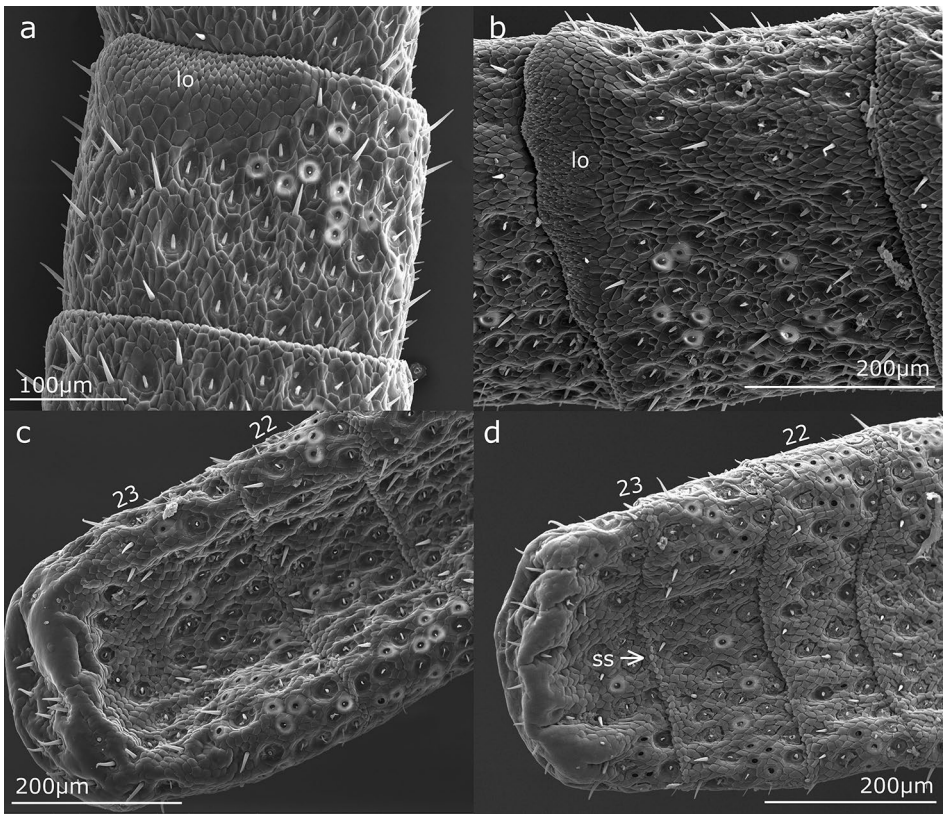

Fig. 3 Antennal morphology of New Zealand alpine grasshoppers (Acrididae; Catantopinae). The lenticular organ (Bland 1989) on the dorsal surface of the 20th segment (**a**) and the ventral surface of the 14th segment (**b**). An example of antennae tip (segment 23) without subsection (**c**) and with subsection (**d**) in *Brachaspis nivalis*. *lo* lenticular organ, *ss* segment subsection. Numbers indicate segment numberings from attachment to head (most proximal segment)

et al. 2005; Ochieng et al. 1998). Preliminary observations showed a similar pattern of sensilla distribution in *B. nivalis*, *S. australis* and *P. nitidus*, so all sensilla on these thirteen distal segments were recorded.

Sensilla were classified according to the nomenclature used for the locusts *Schistocerca gregaria* and *Locusta migratoria* since these are the most extensively studied taxa (Nakano et al. 2022). The number and size of sensilla was

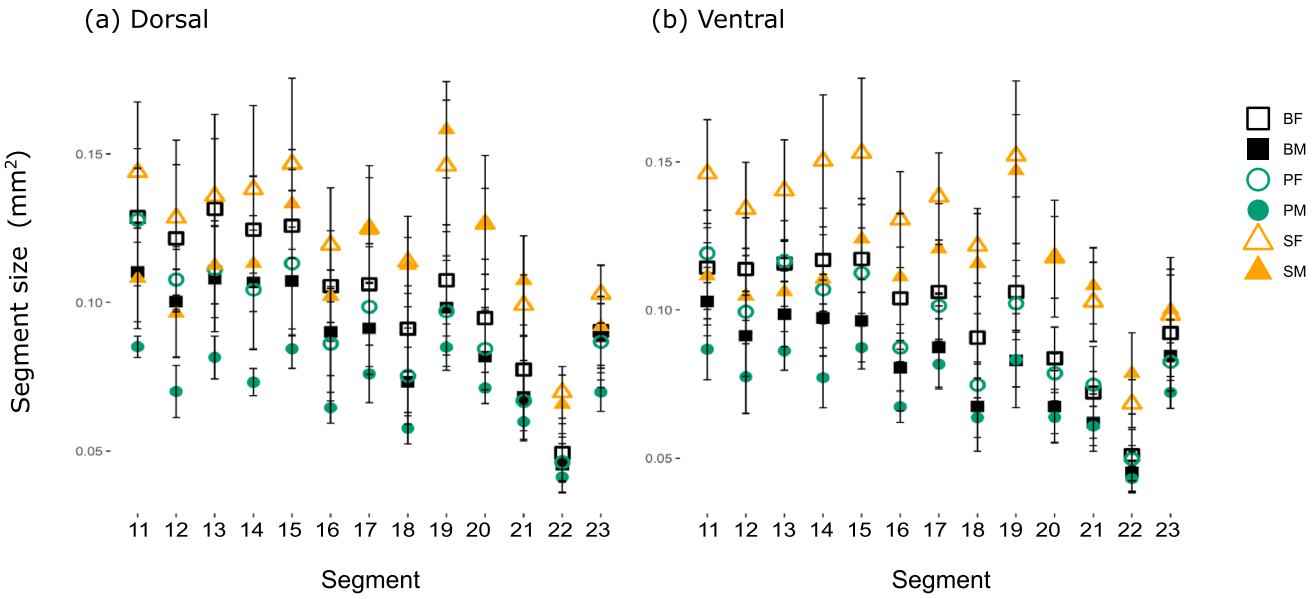

Fig. 4 Variation in antenna size distinguishes three New Zealand alpine grasshopper species. The surface area of each antennal segment (mm^2) for distal half of antennae (segments 11 to 23) on the dorsal (**a**) and ventral (**b**) surfaces. Vertical bars indicate standard deviation. *BF Brachaspis nivalis* female, *BM B. nivalis* male, *PF Paprides nitidus* female, *PM P. nitidus* male, *SF Sigaus australis* female, *SM S. australis* male

counted and measured using the add-in Cell Counter and the Measure functions in Image/Fiji respectively.

Statistical analysis

All statistical analyses were performed in the R statistics environment (R Core Team 2022) using the software platform R Studio 4.0.3 (Boston, MA, USA) and graphics are generated using R Studio 4.0.3 and Inkscape 1.2. Statistical normality was tested by the Kolmogorov–Smirnov test before further analysis. Using a student T-test, the body length (mm), antenna length (mm) and segment area (mm^2) between species of the same sex, and total number and each type of sensilla recorded on the dorsal and ventral surfaces were compared. Differences in total number and number of each type of sensilla on segments 11 to 23 of the dorsal and ventral surfaces were analyzed among species and sexes of the grasshoppers with a linear model using the lm() function. This was followed by post hoc Tukey honest significant differences for multiple pair-wise comparisons using the emmeans package.

Results

Antennal structure (shape, length, area, and segmentation)

In all three species, an irregular arrangement of sharply pointed cuticular plates known as the lenticular organ (Fig. 3a, b) was observed on the dorsal surface of the 20th antennal segment and the ventral surface of the 14th segment. The length of antennae ranged between 4.3 and 9.3 mm, with *S. australis* having the longest antennae (male 6.64 ± 0.65 mm, female 7.80 ± 0.96 mm), and similar lengths observed in *P. nitidus* (male 5.54 ± 0.33, female 7.32 ± 0.63) and *B. nivalis* (male 5.51 ± 0.71 mm, female 6.83 ± 0.96 mm). The surface area of each of the thirteen distal segments (11–23) differed among the three species (Fig. 4). 2D images can potentially underestimate segment area as antennae are not completely flat, in particular, the dorsal surface of *B. nivalis* antennae were often concave (Fig. 3c, d).

No significant difference was observed in the total antennal length between females of *P. nitidus* and *S. australis* ($p = 0.22$), but antennae of female *S. australis* were significantly longer than antennae of *B. nivalis* females ($p = 0.03$) and the antenna of *S. australis* males were significantly longer than antennae of both *P. nitidus* and *B. nivalis* males ($p < 0.01$). Most of the segments were significantly larger in male and female *S. australis* than other species (Fig. 4, Table S1). This is broadly in proportion with their body size as *S. australis* specimens were significantly larger in terms of body length (male 21.04 ± 7.20 mm, female 31.25 ± 2.88 mm) than *P. nitidus* (male 19.08 ± 1.67 mm, female 27.66 ± 2.03 mm) or *B. nivalis* (male 17.68 ± 4.52 mm, female 24.47 ± 2.16 mm). No significant difference in antenna length was observed between *P. nitidus* and *B. nivalis* ($p = 0.20$ in females,

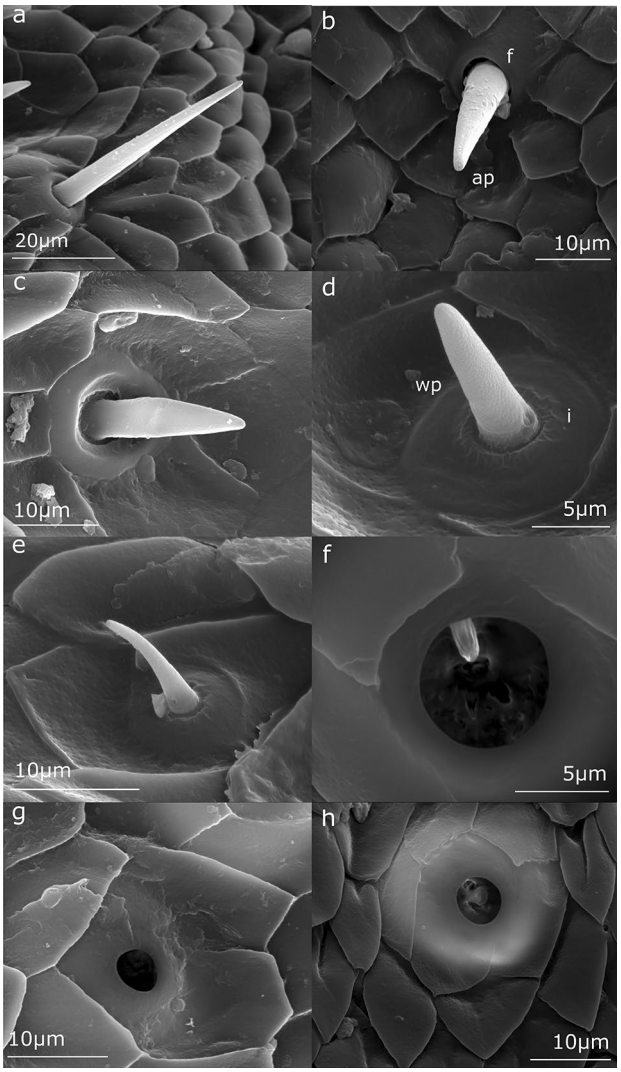

Fig. 5 Types of antennal sensilla found on the antenna of New Zealand alpine grasshoppers (Acrididae; Catantopinae). Chaeticum (**a, b**), basiconicum (**c, d**), trichodeum (**e**), coeloconicum (**f**), cavity sensillum (**g**) and cavity sensillum with internal tissue visible (**h**). *f* flexible socket, *i* inflexible socket, *ap* apical pore, *wp* wall pore

$p = 0.90$ in males) but some segments were significantly larger in both male and female *B. nivalis* compared to *P. nitidus* (Fig. 4, Table S1). In all three species, females had longer antennae (with larger segments) than conspecific males (Fig. 4, Table S1) which is in keeping with their larger body size (Meza Joya et al. 2022).

Sensillatypes, abundance, and distribution

Using sensilla morphology (shape, size, presence/absence of pores, socket types), we recorded five classes of sensilla on the distal antennal segments. These were sensilla chaetica, basiconica, trichoidea, coeloconica and cavity (Fig. 5, Table 1). Within each class of sensilla, size variation was observed (Table 1) and some shape variation was detected in basiconca (Fig. 5c, d), but no species-specific sensilla or shapes were identified. Internal tissue was apparent in images of some cavity sensilla (Fig. 5h) but these were not differentiated from typical cavity sensilla (Fig. 5g).

Males and females of all three species had significantly more chemo-sensilla on the ventral surface of their antennae than on the dorsal surface (Fig. 6a). Three types of olfactory sensilla (basiconica, coeloconica and cavity) were significantly more abundant on ventral surfaces in all species (Fig. 6c, e, f), but significantly more gustatory sensilla (chaetica) were found on the dorsal surfaces of male and female *B. nivalis* antennae (Fig. 6b). No class of sensilla was restricted to a single antennal surface, sex, or species.

The distribution of the five sensilla types along the antennae was consistent among males and females of *B. nivalis*, *S. australis* and *P. nitidus* (Fig. 7). Gustatory sensilla (chaetica) were most abundant at the distal end of each antenna (segment 23) (Fig. 7b, h) in all species. For example, the last antennal segment of *S. australis* had 27–37 chaetica compared to 10–20 on segments 11 to 22 and a similar pattern was seen in *B. nivalis* and *P. nitidus*. Olfactory sensilla consisting of basiconica, coeloconica and cavity were most abundant on the middle antennae segments (especially

Table 1 Types of sensilla, probable function and morphological traits observed on the antennae of three species of New Zealand grasshopper

Sensillum name	Function	Length (µm)	Basal diameter (µm)	Socket type	Pores	Shape
Chaetica (ch)	Gustation & Mechano-reception	15–30	4–6	Flexible	Apical pore	Long peg-like, ribbed wall (Fig. 5a, b)
Basiconica (ba)	Olfaction	10–18	3.5–5.5	Inflexible	Wall-pored	Short and stout peg-like (Fig. 5c, d)
Trichoidea (tr)	Olfaction	11–15	< 3	Inflexible	Wall-pored	Thin hair-like (Fig. 5e)
Coeloconica (co)	Olfaction & Thermo-reception	2.5–3.5	3–10 (pit diameter)	Inflexible	Wall-pored	Peg contained within a pit (Fig. 5f)
Cavity (ca)	Olfaction	N/A	3–10 (pit diameter)	N/A	N/A	Pit with (Fig. 5h) or without (Fig. 5g) visible tissue

Identification of chaetica, basiconica, trichoidea and coeloconica is based on locusts (Altner et al. 1981; Ochieng et al. 1998) and cavity sensilla are based on Chinese grasshoppers (Li et al. 2007)

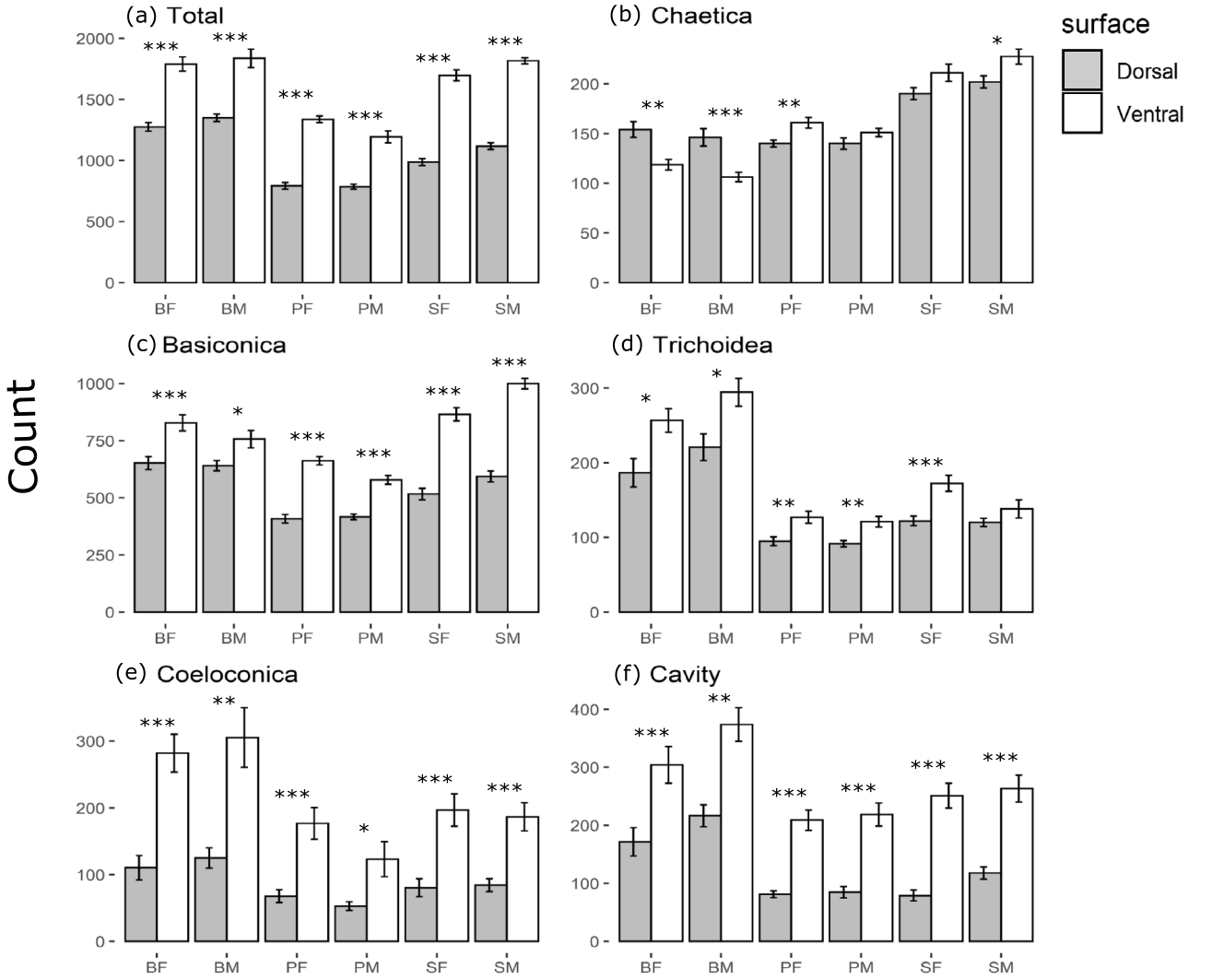

Fig. 6 Mean number of sensilla on dorsal vs. ventral surfaces of distal half of the antennae of three New Zealand alpine grasshopper species (*BF Brachaspis nivalis* female, *BM B. nivalis* male, *PF Paprides nitidus* female, *PM P. nitidus* male, *SF Sigaus australis* female, *SM S. australis* male). Vertical bars indicate standard error. *indicates significant difference of sensilla number between dorsal and ventral surface within a group. Significant level: ***$p<0.001$, **$p<0.01$, *$p<0.05$

on 15 to 20; Fig. 7c, e, f, i, k, l), whereas trichoidea were most abundant on segments 19 or 21 on the dorsal surface (Fig. 7d) and segment 15 on the ventral surface (Fig. 7j).

Comparison of sensilla abundance between species and sexes

The total abundance of sensilla and the proportion of each class on the 13 distal segments of the grasshopper antenna differed between species. *Brachaspis nivalis* had the most chemo-sensilla on their antennae, followed by *S. australis* and *P. nitidus* (Fig. 8a, Table S2). Both male and female *B. nivalis* had significantly more trichoidea than *S. australis* and *P. nitidus* ($p<0.001$) (Fig. 8d) and *B. nivalis* males had significantly more coeloconica ($p<0.02$) and cavity sensilla ($p<0.001$) than the other species (Fig. 8e, f). *Brachaspis nivalis* and *S. australis* had significantly more basiconica than *P. nitidus* ($p<0.001$) (Fig. 8c), and *S. australis* (both males and females) had significantly more chaetica than *B. nivalis* or *P. nitidus* ($p<0.001$) (Fig. 8b).

Female grasshoppers had longer antennae than conspecific males, but no significant differences were observed in the total number of chemo-sensilla between the sexes (Fig. 8a) except for *S. australis* females having fewer basiconica than conspecific males ($p=0.0138$) (Fig. 8c). Although not statistically significant, the *B. nivalis* males examined possessed more olfactory sensilla than their conspecific females (about 15% more trichoidea, 10% more

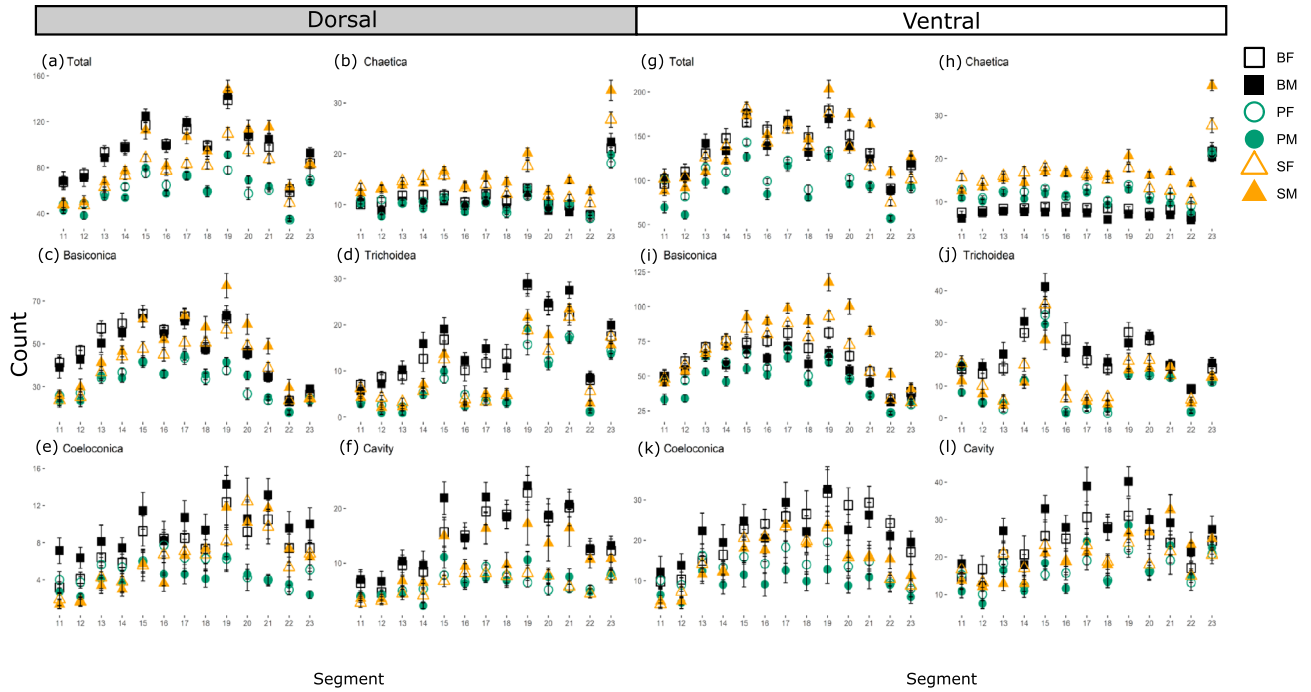

Fig. 7 The distribution of five types of sensilla on the distal half of the antennae of three New Zealand alpine grasshoppers. Mean number of sensilla on the dorsal (**a–f**) and ventral (**g–l**) surfaces is shown for each of 13 segments (segment 23 is antennal tip). Vertical bars indicate standard error. *BF Brachaspis nivalis* female, *BM B. nivalis* male, *PF Paprides nitidus* female, *PM P. nitidus* male, *SF Sigaus australis* female, *SM S. australis* male

coeloconica and 20% more cavity sensilla) while *P. nitidus* females had more olfactory sensilla (about 7% more basiconica and 30% more coeloconica) than their conspecific males (Fig. 8c–f, Table S2).

Discussion

Studies of grasshopper antennal sensilla have focused on particular subfamilies including Gomphocerinae, Oedipodinae and Melanoplinae, and Acridinae. Our comparative study focused on three sympatric and closely related species of Catantopinae. Five classes of chemo-sensilla (chaetica, basiconica, trichoidea, coeloconica and cavity) were identified on the antennae of adult males and females of flightless, alpine, grasshopper species endemic to Aotearoa/New Zealand. The distribution and abundance of sensilla were similar in all three species with sensilla significantly more abundant on the ventral surface of their antennae and chaetica more abundant at their apex. We found that *B. nivalis* had significantly more chemo-sensilla than either *S. australis* or *P. nitidus*. No significant differences in numbers of sensilla were observed between sexes of *B. nivalis* or *P. nitidus*, however, male *S. australis* had more basiconic sensilla than conspecific females.

Sensilla types, abundance, and distribution

Chemo-sensilla are diverse in shape with varying numbers of sensory neurons (Altner et al. 1981; Baker et al. 2008; Jin et al. 2006; Ochieng et al. 1998; Romani and Stacconi 2009; Yang et al. 2012; Zhou et al. 2009) and exhibit sensitivity to different chemical compounds (Altner et al. 1981; Cui et al. 2011; Ochieng and Hansson 1999). Four of the five types of chemo-sensilla present in the grasshopper species examined here (chaetica, basiconica, trichoidea, coeloconica and cavity), have been described and studied in *Schistocerca gregaria* (Cyrtacanthacridinae) and *Locusta migratoria* (Oedipodinae) locusts (Altner et al. 1981; Cui et al. 2011; Jin et al. 2006; Ochieng et al. 1998; Yang et al. 2012; Zhou et al. 2009). In contrast, cavity sensilla were not observed in locusts (Ochieng et al. 1998), but reported from grasshopper species of other subfamilies; *Acrida cinerea* (Acridinae), *Chrysacris changbaishanensis*, *Chrysacris jiamusi*, *Chrysacris heilongjiangensis*, *Chrysacris liaoningensis*, *Mongolotettix angustiseptus*, *Euthystria lueifemora* and *Chrysochraon dispar* (Gomphocerinae) (Li et al. 2007). We identified the rosette of cuticular plates (Bland 1982) or lenticular organ (Bland 1989; Chen et al. 2003), which has previously been recorded on the distal end of antennae in other Acridid species, but its function is unknown.

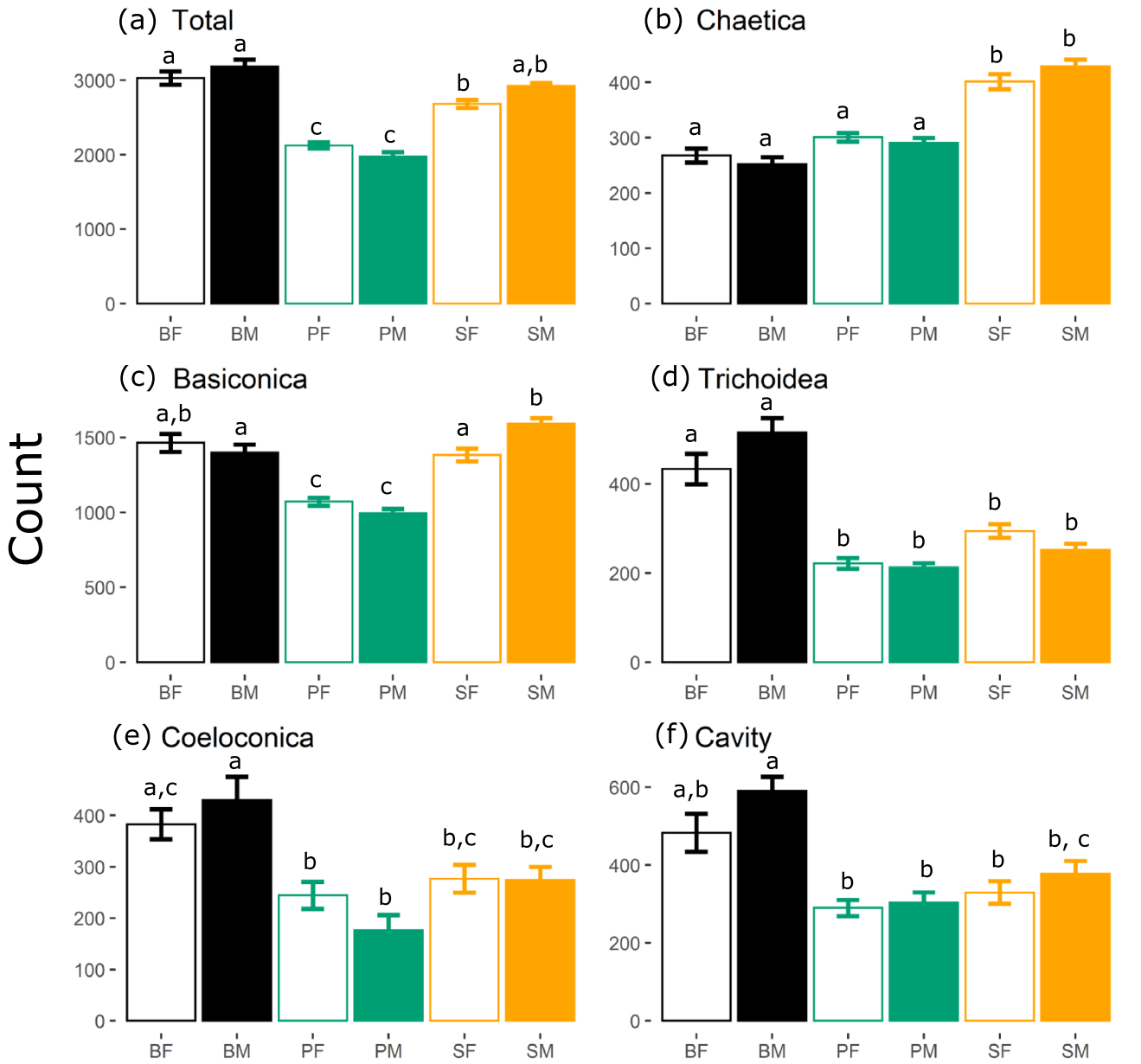

Fig. 8 Variation in abundance of sensilla on the antennae of three New Zealand alpine grasshopper species. Mean number of each sensilla type on the dorsal surface of the distal half of antennae are compared. Vertical bars indicate standard error. Different letters indicate significant differences between males and females within a species revealed from a linear model followed by a pair-wise post hoc Tukey honest significant test (Table S2). *BF Brachaspis nivalis* female, *BM B. nivalis* male, *PF Paprides nitidus* female, *PM P. nitidus* male, *SF Sigaus australis* female, *SM S. australis* male

Sensilla chaetica have contact-chemical and mechano-receptive functions (with their flexible attachments), whereas basiconica and trichoidea are olfactory (Bland 1989; Chen et al. 2003; Cui et al. 2011; Jin et al. 2006; Li et al. 2007; Ochieng et al. 1998). In short-horned grasshoppers, there are two known types of sensilla coeloconica: one with a blunt tipped peg and an apical pore sensitive to temperature and humidity; and the other with a sharp-tipped peg and wall pores sensitive to temperature and olfaction (Altner et al. 1981). Sensilla coeloconica in the New Zealand alpine grasshoppers have a sharp-tipped peg and are therefore likely to be thermo- and olfactory-receptors. Although electrophysiological examination of cavity sensilla has never been made, they are considered to be olfactory since they are distributed in a similar pattern to other olfactory sensilla (Li et al. 2007). Some of the cavity sensilla examined

contained visible internal tissue (Fig. 5h) but these were assumed to be typical olfactory sensilla and were not differentiated (Fig. 5g). This unusual form has not been reported before from grasshoppers, but their detection might simply result from cuticle orientation and high resolution imaging.

We detected size and shape variation within types of sensilla as observed in locusts, where they are interpreted as capable of detecting different chemical stimuli and housing different types of chemosensory neurons and proteins (e.g., chaetica: Zhou et al. 2009; trichoidea: Cui et al. 2011, You et al. 2016), and this may be the case for the alpine grasshoppers studied here.

Few studies have compared the ventral and dorsal surfaces of grasshopper antennae (Bland 1982) or those of other insects (Liu et al. 2021; Romani and Stacconi 2009; Yuvaraj et al. 2018). Comparisons can reveal complex specialization, for example, female mugwort grasshoppers *Hypochlora alba* (Melanoplinae) have 25% more coeloconica on their ventral surface compared to their dorsal surface, but males of the species have 10% more on the dorsal than the ventral surface (Bland 1982). With the exception of chaetica on *B. nivalis* antennae, chemo-sensilla were significantly more abundant on the ventral surface compared to the dorsal surface of all three New Zealand alpine grasshopper species. In live grasshoppers, the ventral surface of the erect antennae face forward, detecting stimuli in front of them (as shown in Fig. 1).

Patterns of sensilla distribution along the antennae of the three New Zealand alpine grasshoppers were broadly similar to observations of other grasshopper species. For example, high abundance in olfactory sensilla (basiconica, trichoidea, coeloconica) at the middle to distal portion has been observed in other species (Bland 1989; Chapman 1989; Li et al. 2007; Ochieng et al. 1998). At the most distal end of the antenna, sensilla chaetica are most abundant, and therefore it is likely that this segment is predominantly involved in gustation (contact chemoreception). Watson (1970) observed New Zealand alpine grasshoppers touching plants with their antennae, suggesting that touch (either mechanical or contact chemoreception) is used for food selection.

Comparison of sensilla abundance between species and sexes

The abundance of sensilla is often linked to species-specific characteristics in the distribution of food and the roles of the two sexes (Bland 1989; Chen et al. 2003; Malo et al. 2004; Li et al. 2007, 2021a; b). Notable in this study was how few species-specific or sex-specific differences we detected. We saw few differences when sensilla of *S. australis* and *P. nitidus* were compared, however, we found that *B. nivalis* have distinct sensilla abundance when compared to *S. australis* or *P. nitidus*. *Brachaspis nivalis* have large distal segments although their antennae are the same length as *P. nitidus*. Enlarged segments at the distal end of antenna facilitate more olfactory sensilla, where *B. nivalis* have significantly more trichoid sensilla than either *S. australis* or *P. nitidus*, and male *B. nivalis* have significantly more coeloconica and cavity sensilla than other species. The number of chaetica (gustatory sensilla) in *B. nivalis* is significantly lower than seen on *S. australis* antennae. These sensilla differences suggest that *B. nivalis* may rely more on olfaction (i.e., distance cues) than gustation (i.e., contact cues) compared to *S. australis*. This is consistent with their association with rocky/scree habitat where food plants are sparser than the habitats of *S. australis* and *P. nitidus* (Bigelow 1967; Koot 2018; Watson 1970). On scree slopes, *B. nivalis* may be more reliant on long-range signals than short-range signals to find food sources. Both *S. australis* and *P. nitidus* are commonly found in mixed shrub, herb and scree habitats than scree-only habitats (Koot 2018; Watson 1970), but *S. australis* (both males and females) have significantly more chemo-sensilla on their antennae than *P. nitidus*. *Paprides nitidus* antennae are also shorter and have significantly smaller segments than those of *S. australis*.

In many grasshopper species, males have more sensilla on their antennae than females (80% of 75 species examined by Bland 1989, Chen et al. 2003 and Li et al. 2007). These sexual differences are attributed to natural selection on males to have high sensitivity to pheromones released by females (Chen et al. 2003; Malo et al. 2004; Wee et al. 2016; Li et al. 2021b). As the New Zealand alpine grasshoppers tend to be visually cryptic (to avoid visual predators) and do not generate acoustic signals with wings when searching for mates (Watson 1970; personal observation), we expect chemical communication to be important in all three species. In the present study, however, the number of sensilla displayed by males and females differed very little. We did find that male *S. australis* had significantly more basiconica than females. Basiconica, also called short basiconica (Bland 1989; Chen et al. 2003) or basiconic sensilla I–V (Li et al. 2007) have been reported as more abundant in males of other species belonging to Melanoplinae, Cyrtacanthacridinae, Oedipodinae, Gomphocerinae, northern Catantopinae, Pamphaginae and Acridinae, in 36/55 species examined by Bland (1989), all 12 species by Chen et al. (2003), and all eight species by Li et al. (2007). Sex-biased abundance of sensilla type may be due to sex-specific requirements to detect particular stimuli, such as sex pheromones and oviposition-site selection (Rai et al. 1997; Chen et al. 2003; Malo et al. 2004; Wee et al. 2016; Roh et al. 2020; Li et al. 2021b).

No significant difference was observed between male and female *P. nitidus* although females usually had more basiconica and coeloconica than males. An equal number of sensilla with similar olfactory sensitivity between sexes observed in *A. barbensis* is thought to reflect their reliance

on visual and auditory cues when finding mates (Chen and Kang 2000). However, solitarious *S. gregaria* males showed higher electrophysiological responses to potential sex pheromones than solitarious females (Ochieng and Hansson 1999) despite the equal abundance of sensilla in males and females (Ochieng et al. 1998). Detailed investigations using neurological and electro-physiological studies are required to further characterize sexual differences in the olfactory sensitivity and functional diversity of sensilla. All three grasshopper species studied here have relatively large eyes, and it is possible that despite their disruptive and camouflage color patterning they signal visually to one another. This study serves as a base for further behavioral and electrophysiological (electroantennography or single sensillum recordings) analysis to elucidate the chemical ecology of endemic New Zealand grasshoppers and contribute to understanding of their evolution and diversity.

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Authors contribution All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Mari Nakano. The first draft of the manuscript was written by Mari Nakano and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Data availability The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Code availability Inkscape 1.0, R Studio 4.0.3.

Declarations

Conflict of interest The author declare that they have no conflict of interest.

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Chapter 5

Food plant odor perception in three species of New Zealand alpine grasshoppers

5.1 Introduction

Plant-derived chemicals vary greatly among plant taxa, which herbivores use to recognize their food (Chapter 2). Chemicals that are commonly found in plants include green leaf volatiles (GLVs) which are either 6-carbon alcohols, aldehydes or esters, terpenoids which are derived from 5-carbon isoprene units, and aromatic compounds which are compounds containing at least one benzene ring (Zhou and Jander 2022; Figure 5.1). In insects, these chemical signals are detected by chemo-sensilla (Chapter 2, 4) that are distributed on the sensory organs like antennae and mouthparts. Using plant chemical cues, insects can perceive food, mates and oviposition site, allowing them to orient towards the resources that are essential for survival and reproduction.

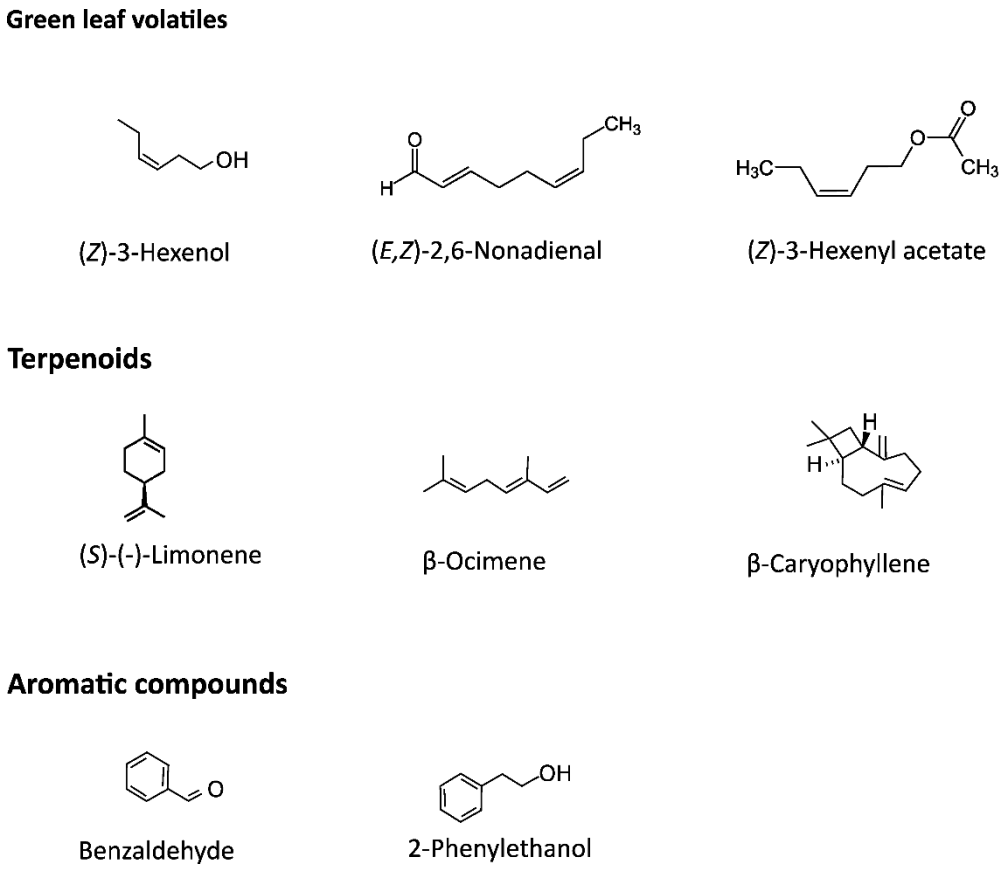

Figure 5.1 Examples of chemicals commonly found in plants.

Observation of insect olfactory responses to their food plants can be explored by analyzing and identifying chemical compounds produced by the plants. Chemical analysis using gas chromatograph coupled with mass spectrometry (GC-MS) can be combined with olfactory responses using electrophysiological tools including gas chromatograph coupled with electroantennographic detection (GC-EAD) and electroantennogram (EAG) (Chen et al. 2004; Seenivasagan et al. 2009; Twidle et al. 2015, 2022). GC-EAD is often used for screening active compounds by exposing insects to a mixture of synthetic compounds or plant extracts (Li et al. 2021) while electroantennogram is used to quantify olfactory responses by exposing insects to individual compounds at different concentrations (Chen et al. 2004; Seenivasagan et al. 2009).

Grasshoppers respond to a variety of plant-derived smells as shown by electrophysiological studies (Blust and Hopkins 1987a, b; Njagi and Torto 1996; Chen and Kang 2000; Chen et al. 2004; Kang and Hopkins 2004). High olfactory sensitivity to food plants can reflect feeding preference in herbivorous insects. For example, the specialist grasshopper *Hypochlora alba* feeds almost exclusively on the brush sage *Artemisia ludoviciana* and show significantly higher electrophysiological responses to terpenoids derived from their host plant compared to the generalist grasshopper *Melanoplus sanguinipes* (Blust and Hopkins 1987a). Sensilla abundance on antennae is also an important factor in determining olfactory sensitivity as higher electrophysiological responses are observed in grasshoppers with more sensilla on their antennae (Blust and Hopkins 1987a; Chen and Kang 2000; Kang and Hopkins 2004).

The sympatric New Zealand alpine grasshoppers, *Brachaspis nivalis*, *Paprides nitidus* and *Sigaus australis*, feed on a variety of plant species including rushes, grasses, dicot herbs, shrubs, ferns and mosses, but have preference towards dicots over monocots (Chapter 3). The morphological types of sensilla in these grasshoppers are very similar, but *B. nivalis* possess

the largest number of olfactory sensilla on their antennae than other species and *S. australis* males possess a higher number of olfactory sensilla than conspecific females (Chapter 4). In this chapter, I explored food choice in captivity, giving wild-caught adult grasshoppers both monocots and dicots plants to eat, and analysed the chemical structures of their food plant volatiles using GC-MS and recorded electrophysiological responses to the smells of their food plants using GC-EAD and EAG. I expected grasshoppers would have the strongest electrophysiological responses to the smells of their favourite food plants, and that *B. nivalis* would respond more strongly than other species and *S. australis* males would respond more strongly than conspecific females due to their higher sensilla abundance.

5.2 Materials and Methods

Insects

Adult grasshoppers and plants were collected at Foggy Peak and Mount Hutt in February 2023 with authority from the New Zealand Department of Conservation (authorization number: 97397-FLO) and ski area operators. Live specimens were transported back to Plant and Food Research (Lincoln, Canterbury) and kept at ambient temperature with natural light with their food plants. These grasshoppers were used for food plant choice tests and electrophysiological analysis.

Food plant choice test

Each grasshopper was introduced to a 6 x 8 x 5 cm plastic container with a mesh top (Figure 5.2A) provisioned with undamaged, fresh leaves from six native plant species: tussock grass *Chionochloa pallens*, rush *Luzula rufa*, the dicot herbs *Gentianella corymbifera* and *Celmisia spectabilis*, and the shrubs *Coriaria sarmentosa* and *Gaultheria crassa*. These plant species

were selected because they are common plant species at Foggy Peak and/or are important components of the diet of New Zealand alpine grasshoppers and represent the spectrum of foliar types available (Chapter 3). Grasshoppers were deprived of food for the night before each 6-hour daytime trial (09:00–15:00), which was run in ambient light and temperature. After each feeding trial, the presence/absence (1/0) of feeding sign (Figure 5.2B) on each plant species was recorded. Nine to 14 trials were performed for males and females of each species.

Figure 5.2 Plastic containers with a choice of six plant species (*Chionochloa pallens, Luzula rufa, Gentianella corymbifera, Celmisia spectabilis, Coriaria sarmentosa* and *Gaultheria crassa* used in the feeding trials (**A**) of New Zealand grasshoppers and the examples of feeding sign (indicated as triangles) observed after a feeding trial (**B**).

Chemical collection and analysis from alpine plants

For chemical extraction, 1g of freshly cut leaf tissue from each of *C. pallens*, *L. rufa*, *G. corymbifera*, *C. spectabilis*, *C. sarmentosa* and *G. crassa* was submerged into a 20 mL of hexane (n = 4) at Foggy Peak 2023. This was kept in a cooler box with ice packs and transported back to the Plant and Food Research lab and kept at a 4 °C fridge for 24 hours. The 24 hours timeframe was used because more varieties of compounds were extracted with longer extraction time in the preliminary analysis (Supplementary Table 5.1). Plant chemicals

extracted in the same way in March 2022 were used for preliminary GC-EAD analysis to identify candidate active compounds, and from alpine plants in Ruahine Ranges (-39.890961,176.018438), Waiouru Military Ground (-39.27924198066611, 175.74863835477996) and Tongariro National Park (-39.197260,175.564351) to compare chemicals to the Foggy Peak plants. Samples were analysed using a Gas Chromatograph – Mass Spectrometer (GCMS-QP2010, Shimadzu Corporation, Kyoto, Japan), using 30 m x 0.32 mm DB-5 capillary columns. Each hexane solution sample was injected in a split mode, with the temperature steps programmed for 3min at 50 °C then incrementally increased to 95 °C at 5 °C/min, 145 °C at 15 °C/min, 180 °C at 10°C/min, and finally 200 °C at 10 °C/min (23.83 minutes total). Compounds were identified by comparing retention times and mass spectra to those in the NIST (National Institute of Standards and Technology) Library 2005.

For comparison of the chemical composition of damaged (hexane extraction) vs. undamaged plants (headspace sampling), volatiles were collected using a 'push-pull' headspace sampling technique (Nakano et al. 2019) from *Celmisia spectabilis*, *C. viscosa* and *C. lyallii* at Broken River Ski Area in February 2021. Above-ground foliage of live plants in nature was enclosed in polyethylene terephthalate (PET) bags (GLAD Large Oven Bags 350 x 500 mm) and carbon-filtered air simultaneously pushed into (0.85 L/min) and pulled out (0.80 L/min) of the PET bag through Teflon tubes. Volatiles were trapped with a 20 mg Hay SepQ adsorbent filter placed at the tip of the outflow tube. Temperature programming used for GC-MS analysis of these samples was the same as detailed above.

Electroantennographic bioassays

Gas chromatography coupled with an electroantennographic detection (GC-EAD) equipped with a DB-5 column (30 m× 0.25 mm ID, J & W Scientific, Folsom, CA, USA) was used to

identify sensitivity to active compounds in New Zealand grasshoppers. Grasshoppers were kept in captivity for up to two weeks before the experiment. In preparation, grasshopper antenna was abscised at the scape or pedicel and then fixed between two glass capillary electrodes by placing the base and tip of the antenna into electrically conductive gel (Signagel®, Parker Laboratories) (Figure 5.3A).

A preliminary analysis used hexane leaf extracts of *C. pallens, G. corymbifera, C. sarmentosa* and *G. crassa* (extracts of *L. rufa* and *C. spectabilis* were not assayed due to time limitation) to identify active compounds derived from grasshopper's food plants. Active compounds from plant extracts were identified by calculating the Kovats retention index on the comparison of their retention times with reference C7 to C21 alkanes (Supelco) on GC-MS and GC-EAD. A mixture of eleven test compounds (concentration of 0.1 mg/mL) that were either found in food plants of these grasshoppers or common in plants (Table 5.1) was then used for response assays. One µl of a test stimulus was injected into the GC-EAD in a splitless mode. The initial oven temperature was at 60 °C held for 0.5 min, then increased to 280 °C at 20 °C/min and held for 10 mins (total run time 21.5 mins). Helium gas was used as a carrier gas at a flow rate of 30 mL/min. This analysis was repeated four times per individual and two to four times per sex of each species.

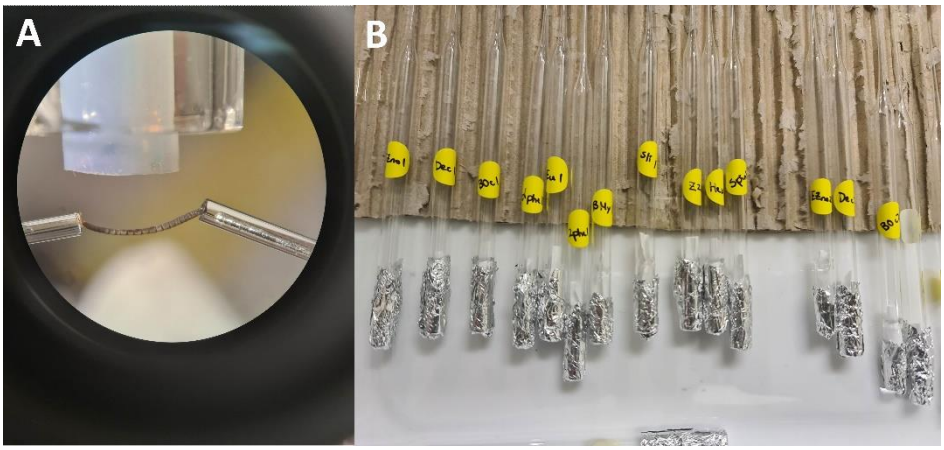

Figure 5.3 Connection of abscised grasshopper antenna between glass capillary electrodes (**A**) and pasteur pipettes loaded with test stimuli (**B**).

Table 5.1 Supplier and purity of 11 compounds used in electroantennographic analysis of New Zealand grasshoppers.

Chemicals	Supplier	Purity
Terpenoids		
(1*S*)-(-)-α-Pinene	Fluka	97%
(*S*)-(-)-Limonene	Sigma Aldrich	96%
α-Phellandrene	Merck-Schuchardt	85%
Eucalyptol	Fluka	99%
β -Myrcene	Sigma Aldrich	90%
β-Ocimene	Sigma Aldrich	90%
Green leaf volatiles		
(*Z*)-2-Hexen-1-ol	Bedoukian	95%
Hexanal	Sigma Aldrich	98%
(*E,Z*)-2,6-Nonadienal	Sigma Aldrich	96%
Decanal	SAFC	98%
Aromatic compounds		
2-Phenylethanol	Sigma Aldrich	99%

Grasshopper electroantennogram (EAG) responses to 11 compounds were recorded by loading 10 µl of each synthetic compound to a piece of filter paper placed into a Pasteur pipette (Sigma-Aldrich; Figure 5.3B). Solvents were allowed to evaporate from the filter paper for 10 seconds under a fume hood before being inserted into the Pasture pipette. These pipettes were used to deliver test stimuli with 0.1 seconds of 'puffs' by a stimulus controller (CS-55, Syntech) into a continuous flow of humidified and charcoal-filtered air stream (600 mL/min) that was flowing over the antennal preparation through a main airflow tube. The dose responses to each of the 11 volatile compounds were measured at doses of 0.1 mg/mL, 1 mg/mL, 10 mg/mL and 100 mg/mL in hexane. β-Caryophyllene (90% purity, Sigma Aldrich) was used as a positive control at a concentration of 10 mg/mL in hexane as this compound elicited stable, consistent responses from the grasshopper antennae. EAG responses to stimuli were recorded sequentially from lower to higher doses and the order of exposure to the 11 compounds at each dose was randomized for each specimen. Hexane and positive controls

were used at the beginning of each dose to ensure no deterioration in the sensitivity of antennae between the doses. An interval of 30 seconds was given between successive test stimuli, as preliminary assays showed this was sufficient for the antenna to recover olfactory sensitivity (Supplementary Figure 5.1). Preliminary observations (Supplementary Figure 5.2) showed that the quantity of (1*S*)-(-)-α-pinene released from Pasteur pipette cartridge decreased more rapidly than other test compounds, so test pipettes were refreshed with newly treated filter papers after five puffs for (1*S*)-(-)-α-pinene and after ten puffs for other compounds.

Statistical analysis

Because insects used in this study were of unknown adult age and mating status, which could potentially affect their olfactory sensitivity (Onagbola and Fadamiro 2011; Gadenne et al. 2016), normalised responses were used for the analysis. This was calculated by dividing the absolute EAG response (mV) to a given test compound by the EAG response (mV) to a positive control (i.e., β-caryophyllene at 100 mg/mL). A positive control instead of a hexane control was used to normalize as it gave consistent responses by insects. All statistical analyses were performed in the R statistics environment (R Core Team 2023) using the software platform R Studio 4.0.3 (Boston, MA, USA) and graphics were generated using R Studio 4.0.3 and Inkscape 1.2.2. The statistical normality of data was tested by Shapiro-Wilk test. To test for significant differences in normalized EAG responses among 11 test stimuli among grasshopper species and sexes, data were treated to Analysis of variance (ANOVA) followed by Tukey's Honest Significant Test.

5.3 Results

Feeding choice test

Among six tested plant species provided to them in captive trials, adult alpine grasshoppers showed clear preferences. The shrub *Coriaria sarmentosa* was highly favoured with >91% of male *B. nivalis*, 100% of female *B. nivalis* and all *P. nitidu*s and *S. australis* feeding on it. This contrasted with none of the grasshoppers feeding on the tussock grass *Chionochloa pallens* (Figure 5.4). The dicot herb *Gentianella corymbifera* was also used in the trials with feeding by >60% of female *P. nitidus* and *S. australis*, >50% of *B. nivalis* males, >40% of *S. australis* males and *B. nivalis* females and 20% of male *P. nitidus*. The rush *Luzula rufa* and the shrub *Gaultheria crassa* were fed to a lesser degree with one each of *S. australis* and *B. nivalis* males eating both plant species, and the other classes to a lesser extent. *Celmisia spectabilis* was fed only by *P. nitidus* females and *S. australis* females.

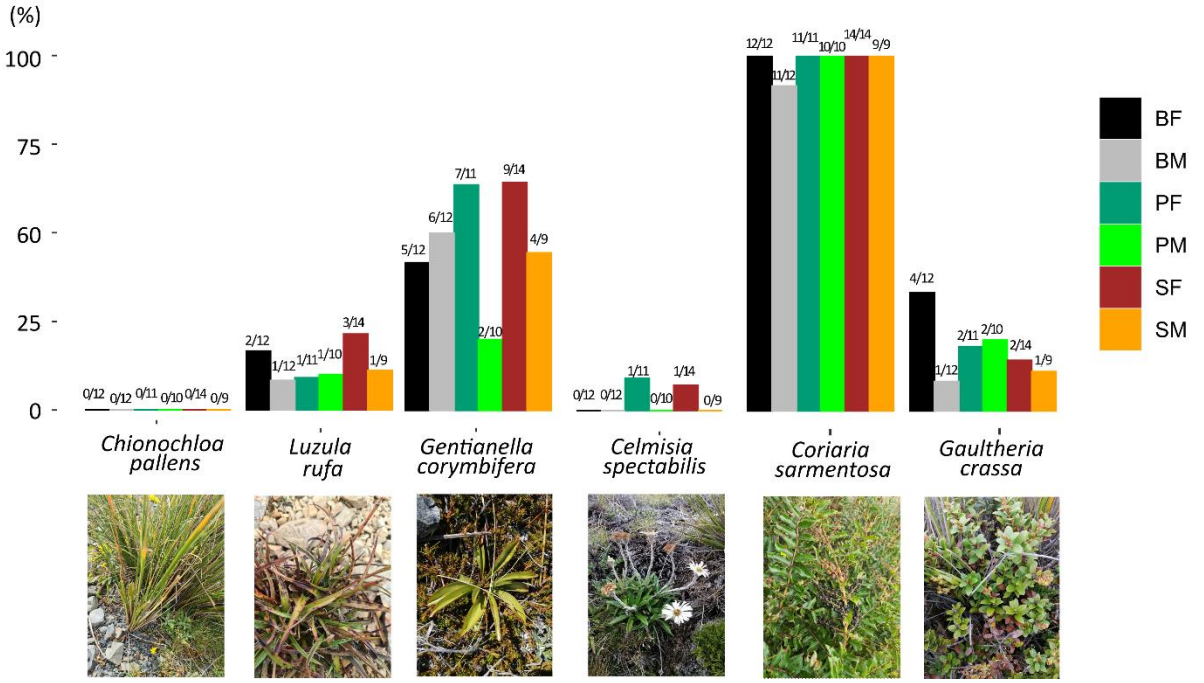

Figure 5.4 Percentage of individuals (n = 9–14) fed on each plant species, tussock grass *Chionochloa pallens*, rush *Luzula rufa*, dicot herbs *Gentianella corymbifera* and *Celmisia spectabilis*, and shrubs *Coriaria sarmentosa* and *Gaultheria crassa*. Numbers above each bars are (number of individuals fed on plant) / (sample size). Abbreviations: BF = *Brachaspis nivalis* female; BM = *B. nivalis* male; PF = *Paprides nitidus* female; PM = *P. nitidus* male; SF = *Sigaus australis* female; SM = *S. australis* male.

Plant chemicals and active compounds

Eighteen compounds were detected from the leaf extracts of *Chionochloa pallens*, *Luzula rufa*, *Gentianella corymbifera*, *Celmisia spectabilis*, *Coriaria sarmentosa* and *Gaultheria crassa* (Table 5.2). α-Pinene, limonene, hexanal and (*E,Z*)-2,6-nonadienal were detected in all six plant species. The chemicals detected were different between solvent extraction and head-space collection. Six terpenoids, 11 green leaf volatiles and one ketone were found in leaves from hexane extracts (Table 5.2) while 11 terpenoids, two green leaf volatiles and one aromatic compound were detected in the headspace collection of three *Celmisia* species (Supplementary Table 5.2). More varieties of green leaf volatiles than terpenoids or other chemical classes were also found in the hexane extracts of alpine plant leaves from Ruahine

Ranges, Waiouru Military Ground and Tongariro National Park (Supplementary Tables 5.3–5.5). Some compounds identified from hexane extraction and headspace collection were used for further EAG analysis.

Table 5.2 Chemical compounds found in leaf extracts of alpine plants in Foggy Peak. Compounds in bold are the compounds used for electrophyisiological analysis.

Chemicals	*Chionochloa pallens*	*Luzula rufa*	*Gentianella corymbifera*	*Celmisia spectabilis*	*Coriaria sarmentosa*	*Gaultheria crassa*
Terpenoid						
α-Pinene	+	+	+	+	+	+
Limonene	+	+	+	+	+	+
Eucalyptol			+			
cis-Geraniol			+		+	
Phytol						+
Copaene						+
Green leaf volatile						
Hexanal	+	+	+	+	+	+
(*E*)-3-Hexen-1-ol	+		+	+	+	+
(*Z*)-3-Hexen-1-ol	+		+	+	+	+
(*Z*)-2-Hexen-1-ol	+	+			+	+
cis-3-Hexenyl acetate	+	+				
4,8-dimethyl-1-Nonanol			+	+		
(*Z,Z*)-3,6-Nonadienal	+	+	+			
Nonanal	+			+	+	+
2-propyl-1-Heptanol	+					
(*E,Z*)-2,6-Nonadienal	+	+	+	+	+	+
2-Hexyl-1-octanol	+		+		+	+
Ketone						
2-Heptanone				+	+	+

GC-EAD analysis using leaf extracts showed grasshoppers responded consistently to the compounds with the same retention times regardless of the plant species used (Figure 5.5). These six compounds were identified to be the green leaf volatiles, (*Z*)-2-hexen-1-ol, hexanal, nonanal, (*Z*,*Z*)-3,6-nonadienal, (*E*,*Z*)-2,6-nonadienal, and 2-hexyl-1-octanol, respectively.

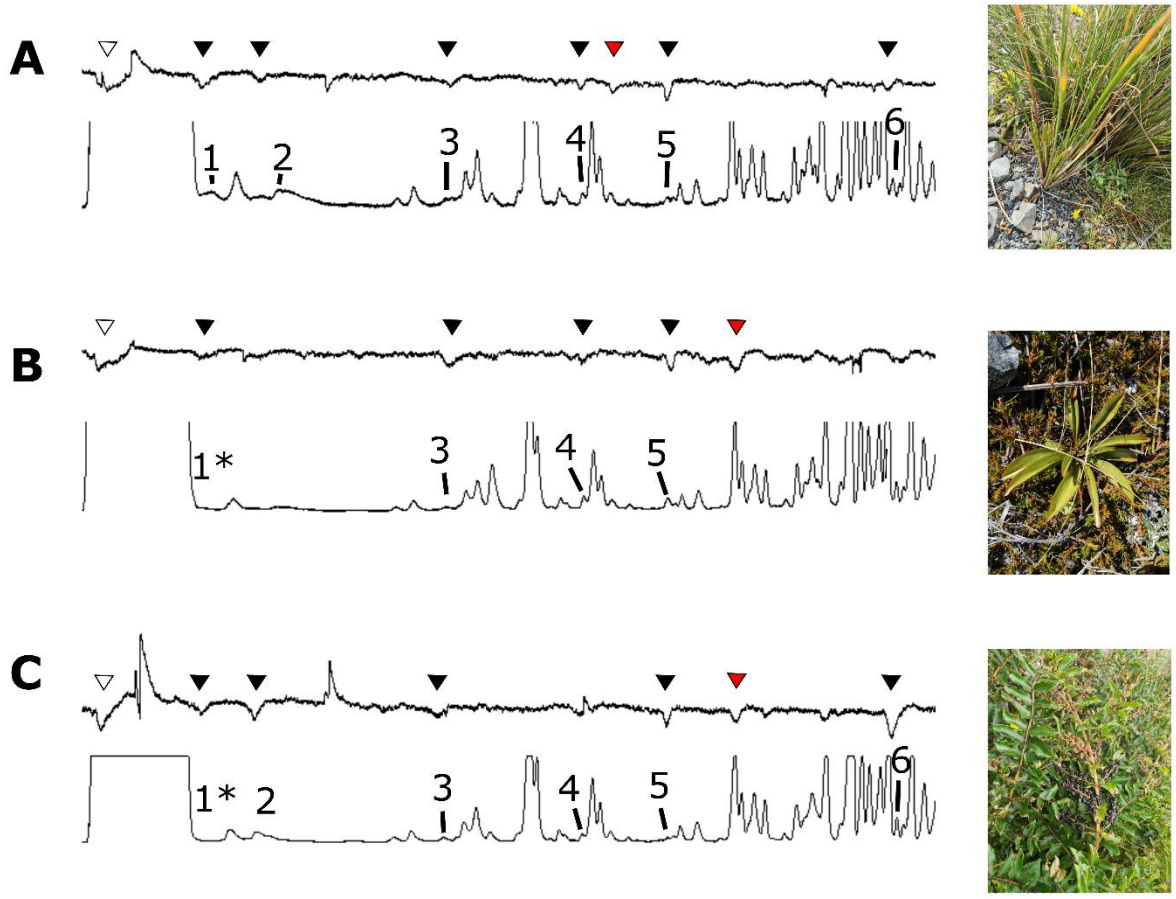

Figure 5.5 GC-EAD recordings in New Zealand alpine grasshopper to hexane leaf extracts of *Chionochloa pallens* (**A**), *Gentianella corymbifera* (**B**) and *Coriaria sarmentosa* (**C**). Olfactory responses are indicated as triangles; empty for response to hexane, black for identified compound, and red for unidentified compound. Peak numbers: 1 = (*Z*)-2-hexen-1-ol, 2 = hexanal, 3 = nonanal, 4 = (*Z*,*Z*)-3,6-nonadienal, 5 = (*E*,*Z*)-2,6-nonadienal, 6 = 2-hexyl-1-octanol. *indicates compound present in trace amounts.

The GC-EAD analysis using a mixture of synthetic compounds showed that both sexes of all three grasshopper species responded to (*E*,*Z*)-2,6-nonadienal and decanal at 0.1 mg/mL (peak

10 & 11 in Figure 5.6). Males and females of *B. nivalis* and some *S. australis* and *P. nitidus* individuals also showed responses to two green leaf volatiles (*Z*)-2-hexen-1-ol and hexanal, and to an aromatic compound 2-phenylethanol (peak 1, 2, 9 in Figure 5.6). The only terpenoid compound that elicited a response from grasshopper antennae was β-ocimene (peak 8 in Figure 5.6). *Brachaspis nivlais* also showed responses to a compound present as an impurity in the test solution (indicated as blue triangles in Figure 5.6), which was subsequently identified as a terpenoid 2,6-dimethyl-2,4,6-octatriene.

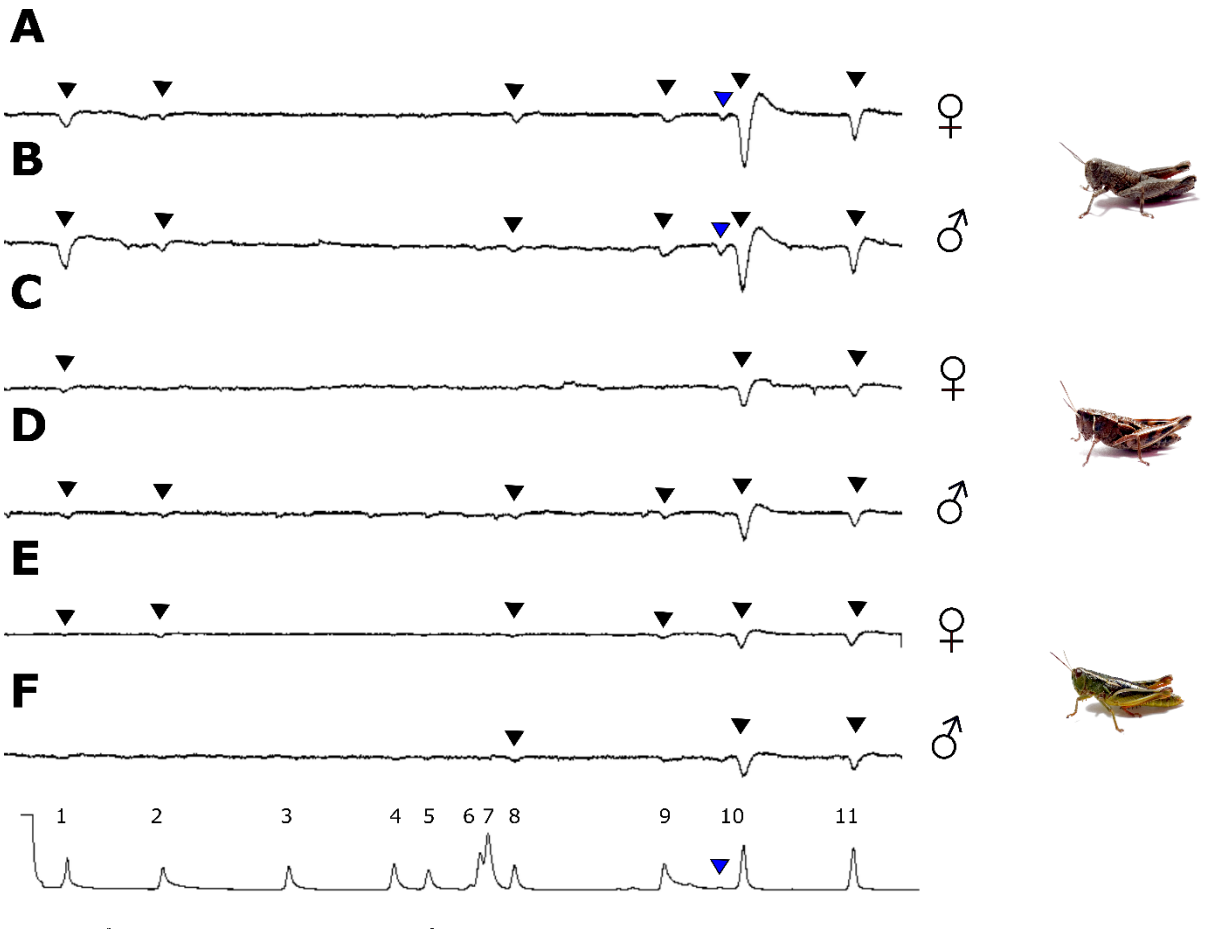

Figure 5.6 Active compounds (indicated as black triangles) identified by gas chromatograph coupled with antennographic detection (GC-EAD) in *Brachaspis nivalis* female (**A**) and male (**B**), *Sigaus australis* female (**C**) and male (**D**), *Paprides nitidus* female (**E**) and male (**F**). Blue arrows indicate grasshopper response to 2,6-dimethyl-2,4,6-octatriene, which was not included in the synthetic mixture. Peaks 6 and 7 are co-eluting. Chemical peaks: 1 = (*Z*)-2-hexen-1-ol, 2 = hexanal, 3 = (*1S*)-(-)-α-pinene, 4 = β-myrcene, 5

= α-phellandrene, 6 = eucalyptol, 7 = (*S*)-(-)-limonene, 8 = β-ocimene, 9 = 2-phenylethanol, 10 = (*E,Z*)-2,6-nonadienal, 11 = decanal.

Electroantennogram (EAG)

All individuals responded to (*E,Z*)-2,6-nonadienal at the lowest concentration (0.1 mg/mL) (Figure 5.7A; Table 5.3). At this same low concentration (0.1 mg/mL) all *B. nivalis* males and >80% of females responded to other green leaf volatiles, and >50% of males and females of the same species showed responses to all terpenoids and 2-phenylethanol except for (1*S*)-(-)-α-pinene which elicited responses from 30% of females. Most terpenoids elicited responses from <50% of the *P. nitidus* and *S. australis* individuals except 50% of *P. nitidus* females responded to β-ocimene. >50% of males and females of *S. australis* showed responses to (*Z*)-2-hexen-1-ol and hexanal and >50% of *P. nitidus* showed responses to (*Z*)-2-hexen-1-ol. >50% of *P. nitidus* and *S. australis* females showed responses to decanal. None of the males and females of *P. nitidus* showed responses to 2-phenylethanol at 0.1 mg/mL and none of *P. nitidus* females showed responses to (*S*)-(-)-limonene at the same concentration.

Table 5.3 Proportion (as %) of individual grasshoppers that displayed an EAG resoponse to leaf compounds at a concentration of 0.1 mg/mL in hexane. Shading indicates 100% dark, >50% mid, <50% white.

	B. nivalis		*P. nitidus*		*S. australis*	
	F	M	F	M	F	M
Sample size	6	6	6	6	6	5
Terpenoid						
α-Phellandrene	50	67	17	17	33	20
β-myrcene	67	83	17	17	17	40
β-Ocimene	67	83	50	17	33	20
Eucalyptol	50	100	17	33	33	20
(*S*)-(-)-limonene	50	83	0	33	33	40
(1*S*)-(-)-α-Pinene	30	67	17	17	33	20
Green leaf volatile						
Decanal	83	100	50	33	67	40
(*E,Z*)-2,6-Nonadienal	100	100	100	100	100	100

Hexanal	83	100	33	33	50	80
(*Z*)-2-hexen-1-ol	83	100	67	50	100	80
Aromatic compound						
2-Phenylethanol	67	50	0	0	67	40

All grasshoppers responded to every test stimulus at the highest concentration of 100 mg/mL (Figure 5.7B). All grasshoppers showed more than twice or thrice the average normalized responses to green leaf volatiles, (*E,Z*)-2,6-nonadienal, decanal, (*Z*)-2-hexen-1-ol and hexanal, compared to most of the terpenoids (Figure 5.7B; Supplementary Table 5.6). Their responses to 2-phenylethanol were similar to that of green leaf volatiles (Figure 5.7B).

Grasshopper responses to some compounds were inconsistent across different concentrations. For example, grasshopper responses to low concentration (0.1 mg/mL) of (*E,Z*)-2,6-nonadienal were stronger than those of (*Z*)-2-hexen-1-ol (Figure 5.7A) but the situation was reversed at high concentration of 100 mg/mL (Figure 5.7B).

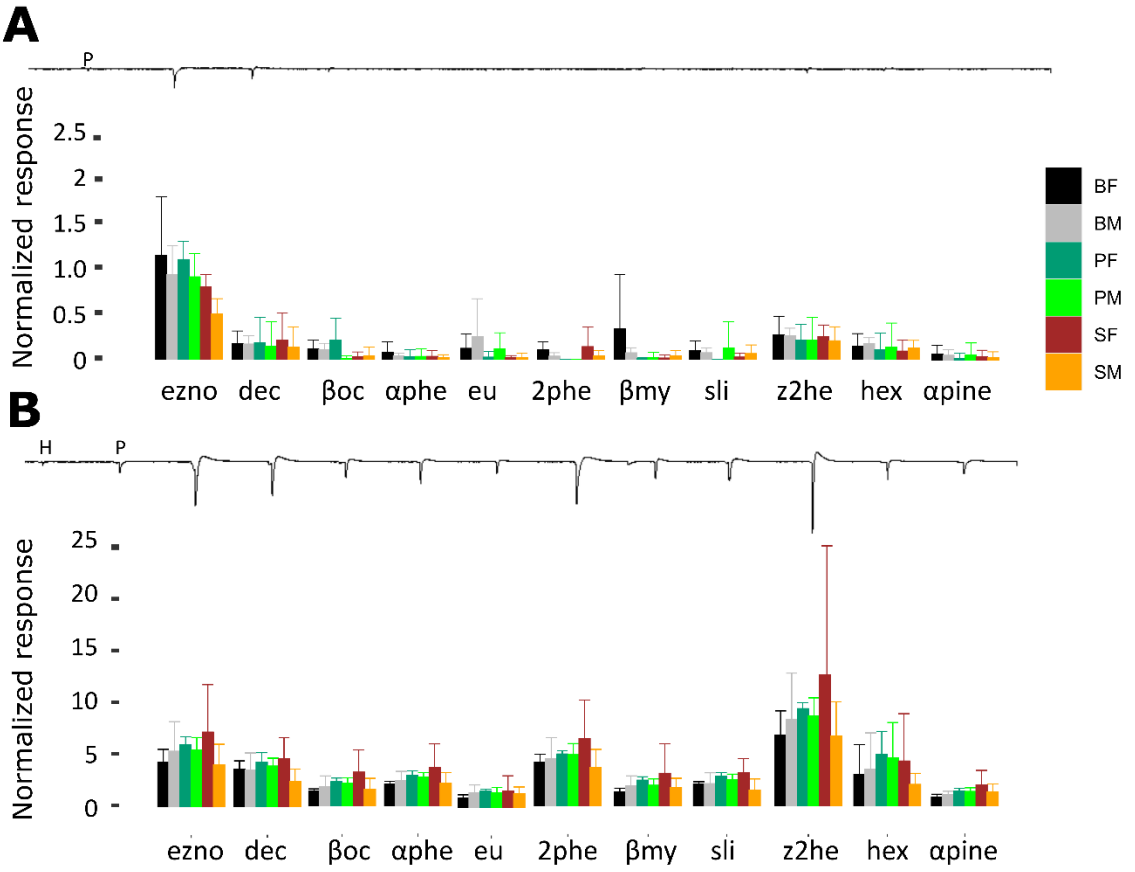

Figure 5.7 Electroantennogram (EAG) recordings and normalized responses (absolute EAG in relation to positive control) of New Zealand alpine grasshoppers to 11 plant volatiles at concentrations of 0.1 mg/mL (**A**) and 100 mg/mL (**B**) observed. The traces show the first two responses (left side) are to hexane control (H) and positive control (P) β-caryophyllene, at 100 mg/mL of hexane. Error bars are standard deviations. Abbreviations: ezno = (*E*,*Z*)-2,6-nonadienal, dec = decanal, βoc = β-ocimene, αphe = α-phellandrene, eu = eucalyptol, 2phe = 2-phenylethanol, βmy = β-myrcene, sli = (*S*)-(-)-limonene, z2he = (*Z*)-2-hexen-1-ol, hex = hexanal, αpine = (1S)-(-)-α-pinene; BF = *Brachaspis nivalis* female; BM = *B. nivalis* male; PF = *Paprides nitidus* female; PM = *P. nitidus* male; SF = *Sigaus australis* female; SM = *S. australis* male.

Significant differences among the grasshopper species and sexes in the normalized responses were observed to some of the test compounds at different concentrations. *Brachaspis nivalis* females showed significantly higher responses to (*E*,*Z*)-2,6-nonadienal at 0.1 mg/mL than those of *S. australis* males (Figure 5.8A). *Paprides nitidus* males showed significantly higher responses to (*Z*)-2-hexen-1-ol at 1 mg/mL than those of *S. australis* males (Figure 5.8B), and to decanal at 10 mg/mL than those of males of *B. nivalis* and *S. australis* (Figure 5.8D).

Paprides nitidus females showed significantly higher responses to α-phellandrene and decanal at 10 mg/mL than those of *S. australis* males (Figure 5.8C, E).

Between sexes, significantly higher responses to (*S*)-(-)-limonene at 100 mg/mL were observed in *S. australis* females than those of conspecific males (Figure 5.8E). However, no significant sexual differences were observed in normalized EAG responses of *B. nivalis* or *P. nitidus* to any of the compounds tested.

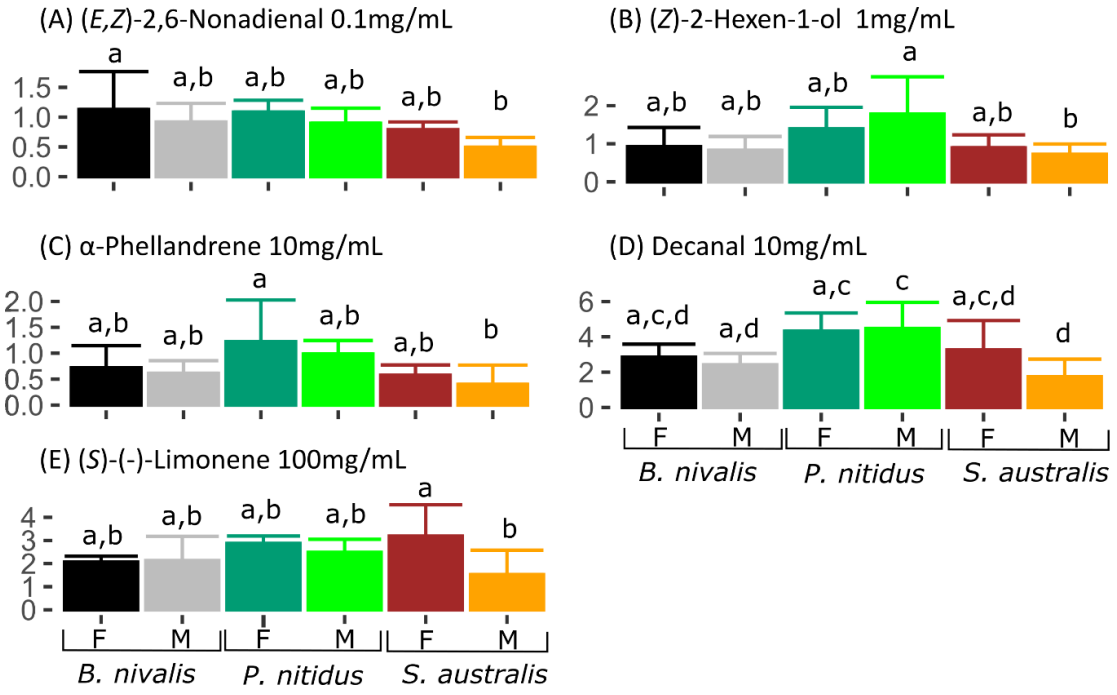

Figure 5.8 Normalized electroantennogram (EAG) responses (absolute EAG in relation to positive control) to synthetic compounds in males and females of New Zealand alpine grasshoppers. Lowercase letters on bars indicate significant differences in responses between species or sexes revealed from the analysis of variance followed by a pair-wise post hoc Tukey honest significant test.

Among the studied concentration range (0.1 mg/mL–100 mg/mL), EAG responses of the grasshoppers increased in a dose-dependent manner (Figure 5.9). Decrease or no change in *P. nitidus* male and females' responses to decanal and (*E,Z*)-2,6-nonadienal was observed when the concentration increased from 10 mg/mL to 100 mg/mL (Figure 5.9I, J).

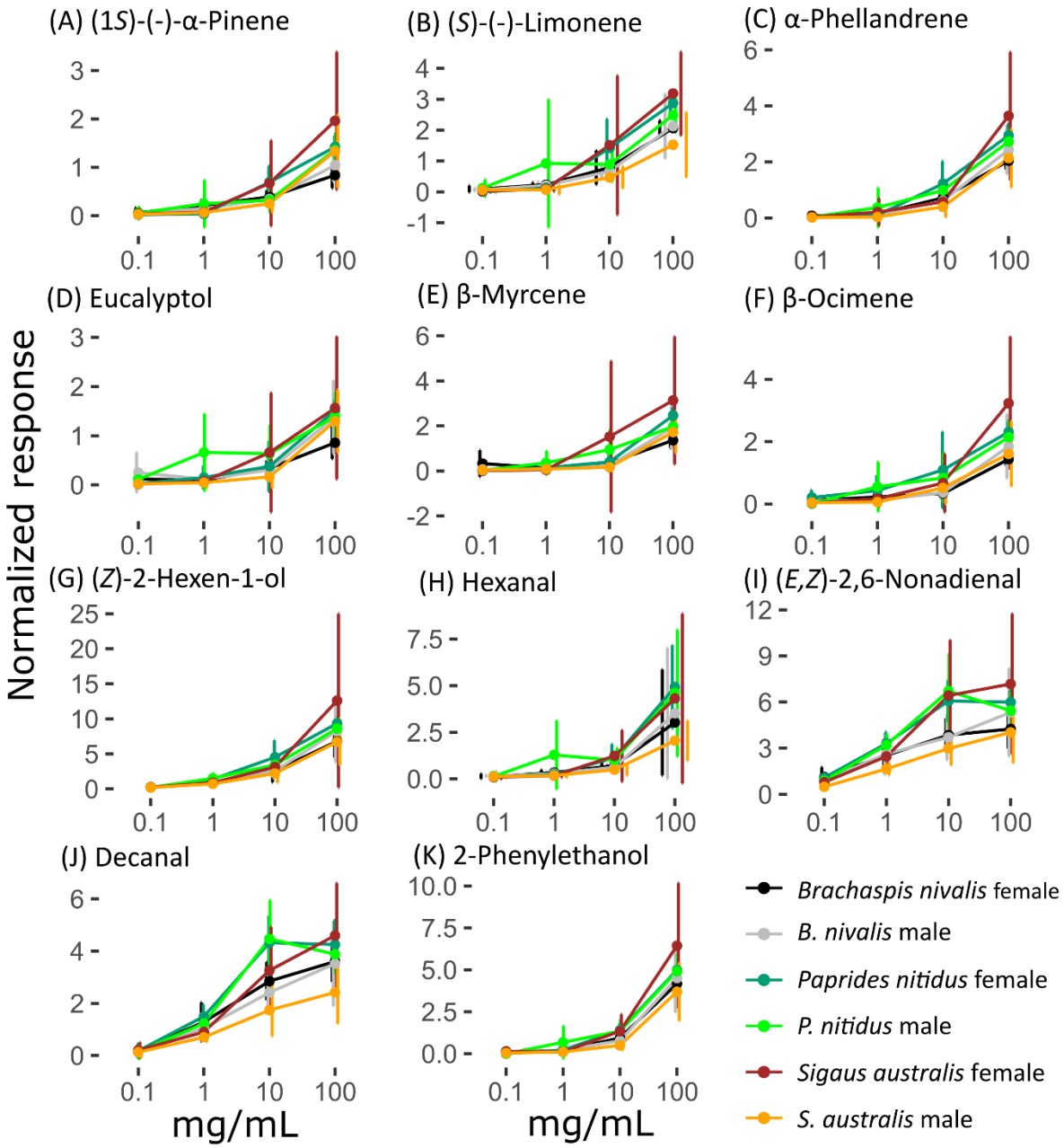

Figure 5.9 Electroantennogram (EAG) responses (mV) relative to positive control in males and females of New Zealand alpine grasshoppers to 11 volatile compounds at four dose ranges (0.1 mg/mL, 1 mg/mL, 10 mg/mL and 100 mg/mL).

5.4 Discussion

Insects are known to show olfactory responses to the smells of their food plants (Njagi and Torto 1996; Chen and Kang 2000; Chen et al. 2004; Kang and Hopkins 2004; Twidle et al. 2015, 2022). High olfactory responses are predicted for preferred food plants compared to less preferred or nonhost plants, for successful food location. In this chapter, I explored feeding preferences among six alpine plant species and recorded electrophysiological responses to the chemicals found in these plant species in New Zealand alpine grasshoppers.

Feeding test

Among six plant species, the shrub *Coriaria sarmentosa* was the most favoured and the grass *Chionochloa pallens* was the least favoured plant in males and females of three alpine grasshopper species. The dicot herb *Gentianella corymbifera* was the second most favoured (20% in *P. nitidus* males and >40% in *P. nitidus* females and other species), which was followed by the shrub *Gaultheria crassa* and the rush *Luzula rufa* which were fed on by 8–30% of individuals of all species, and the herb *Celmisia spectabilis* which was fed on only by >10% of females of *S. australis* and *P. nitidus*. Clear species or sex specific preference towards particular plants was not observed and all individuals preferred *C. sarmentosa* the most regardless of species and sexes. This could be because *C. sarmentosa* produces fruits (Figure 5.10) which are available throughout the year and thus grasshoppers are targeting these as good nutritious sources.

Figure 5.10 *Coriaria sarmentosa* with fruits at Foggy Peak (**A**) and *Brachaspis nivalis* (**B**) and *Sigaus australis* (**C**) feeding on *C. sarmentosa* fruits in captivity.

Food plant and active compounds

Gas chromatograph coupled with electroantennographic detection (GC-EAD) and electroantennogram (EAG) analyses showed that New Zealand alpine grasshoppers are highly sensitive to green leaf volatiles. GC-EAD with leaf extracts showed olfactory responses to several green leaf volatiles ((*Z*)-2-hexen-1-ol, hexanal, nonanal, (*Z,Z*)-3,6-nonadienal, (*E,Z*)-2,6-nonadienal, and 2-hexyl-1-octanol), and similarly, several synthetic compounds were always detected even at the lowest concentration ((*E, Z*)-2,6-nonadienal and decanal). Only one terpenoid (β-ocimene) gave a response from grasshopper antennae in the GC-EAD analysis with a mixture of synthetic compounds, and EAG responses to green leaf volatiles were twice or thrice higher than most terpenoids at the high concentration. Higher

sensitivity to green leaf volatiles than to terpenoids has also been observed in generalist grasshoppers including adult *Oedaleus decorus asiaticus* and *Angaracris barabensis* (Chen et al. 2004) and *Schistocerca gregaria* nymphs (Njagi and Torto 1996) regardless of dicot or monocot feeding habits. On the other hand, the specialist grasshopper *Hypochlora alba* responded to terpenoids found in their host plant *Artemisia ludoviciana* even at very low concentration (0.1 ng/µl: 1/1000 of the lowest concentration used in this study; Blust and Hopkins 1987a). Indeed, most of the compounds found in *A. ludoviciana* elicited significantly higher responses from *H. alba* than those of a generalist grasshopper *Melanoplus sanguinipes*. *M. sanguinipes* showed a significantly higher EAG response to geraniol which is the compound that occurs more commonly in many plants (also detected in this study) compared to other terpenoid compounds found in *A. ludoviciana*. Thus, grasshoppers may show a major difference in their olfactory sensitivity between generalists and specialists.

In all plants, green leaf volatiles are emitted immediately after plants are damaged (Conchou et al. 2019; Zhou and Jander 2022), and more varieties of green leaf volatiles were detected in New Zealand alpine plants from the hexane extract (Table 5.2; Supplementary Tables 5.3–5) than those from headspace collection (Supplementary Table 5.2), possibly because the former method involves damaging plant tissues. Behavioural observations using a Y-maze olfactometer showed smells of damaged plants attracted significantly more than smells of undamaged plants in polyphagous grasshoppers *S. gregaria* (Njagi and Torto 1996) and *M. sanguinipes* (Hopkins and Young 1990; Kang and Hopkins 2004). It is yet to be known whether green leaf volatiles act as attractants or repellents in New Zealand grasshoppers and this needs further behavioural studies.

Between species and sexes

Most of the New Zealand alpine grasshoppers showed enhanced sensitivity to test stimuli as the dose increased, except for *P. nitidus* responses to decanal and (*E,Z*)-2,6-nonadienal decreased or no difference was observed when the concentration increased from 10 mg/mL to 100 mg/mL. The reason behind this is unknown but it is unlikely that the decreased sensitivity to these compounds is associated with decreased sensitivity of the antenna as the sensitivity was ensured with exposure to controls. It is possible that the maximum responses to these compounds were reached at the concentration of 10mg/mL in *P. nitidus*.

All grasshoppers were able to detect every 11 compounds at 100 mg/mL but more individuals of *B. nivalis* showed responses to the test stimuli at a lower dose (0.1 mg/mL) compared to other species (Table 5.2). This result may show that *S. australis* and *P. nitidus* are less sensitive to compounds at smaller doses compared to *B. nivalis*. As low dose sensitivity could be related to long-distance sensitivity (Chen et al. 2004), *B. nivalis* may be more reliant on long-distance cues compared to other species which could be related to their less vegetated rock/scree habitat (Watson 1970; Koot 2018).

Higher sensitivity to external stimuli is considered with a higher abundance of sensilla (Chapter 4), and a higher abundance of sensilla and olfactory response in males than conspecific females were observed in grasshopper *O. decorus asiaticus* (Chen et al. 2004) and *H. alba* (Blust and Hopkins 1987a). In contrast, *S. australis* males were less sensitive to test stimuli than the females although higher sensilla abundance was observed in *S. australis* males than in conspecific females (Chapter 4). The reason behind this is unclear, but it is possible that sensilla abundance is not related to olfactory sensitivity in New Zealand alpine grasshoppers. Alternatively, *S. australis* males may have specific olfactory sensitivity that is

different from conspecific females or other species to the compounds that were not tested in this study.

Although the alpine grasshoppers showed a preference for specific plant species, the chemicals found in the six plant species examined were similar and it is yet to be known how these grasshoppers recognize their favourite food. Only the leaf volatiles were examined in this study and the grasshoppers may use volatiles of other plant parts (e.g., flowers or fruits) to recognize and locate their favourite food. Taste can also be involved in the food selection of the grasshoppers, as seen in other grasshoppers including *H. alba*, *M. sanguinipes* (Blust and Hopkins 1987b), *Chorthippus* species (Picaud et al. 2003), *Bootettix argentatus*, *Ligurotettix coquilletti*, and *Cibolacris parviceps* (Chapman et al. 1988). Macronutrients are important factors in determining food, and one study showed time spent palpating on filter paper loaded with sucrose and fructose was significantly different among four *Chorthippus* grasshopper species (Picaud et al. 2003). Structural aspects of plants (i.e., size, toughness: Chapter 3) as well as visual cues including colours (Picaud et al. 2002) and branching patterns (Picaud et al. 2003) of host plants can also be important factors in discriminating host plants in grasshoppers. Altogether, further studies of chemical, structural and visual cues involved in the food plant selection of New Zealand alpine grasshoppers are required.

5.5 References

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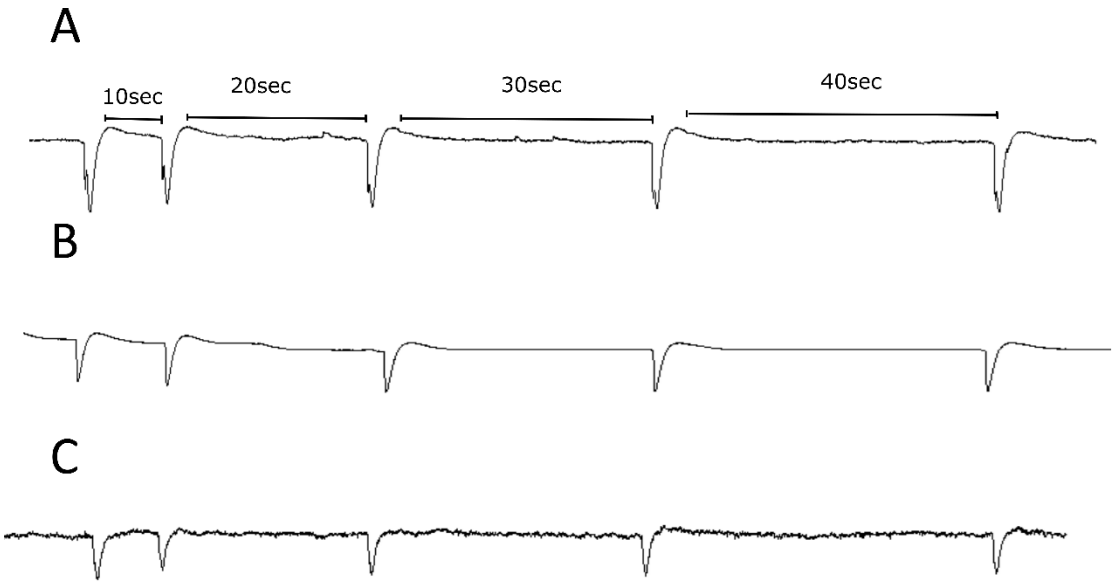
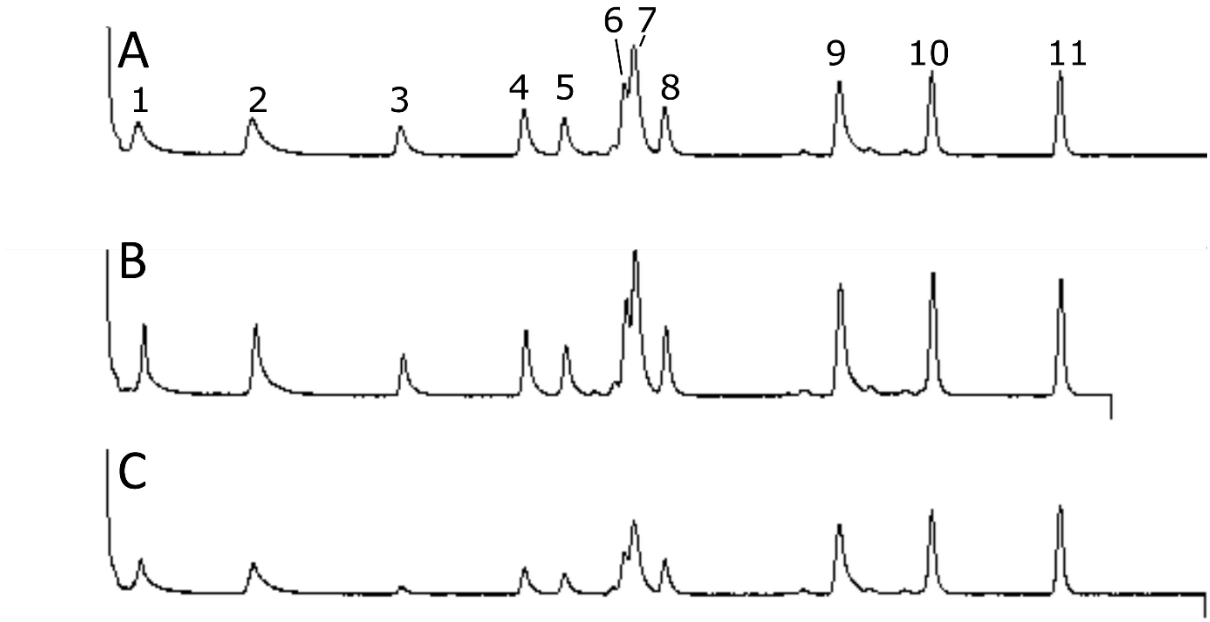
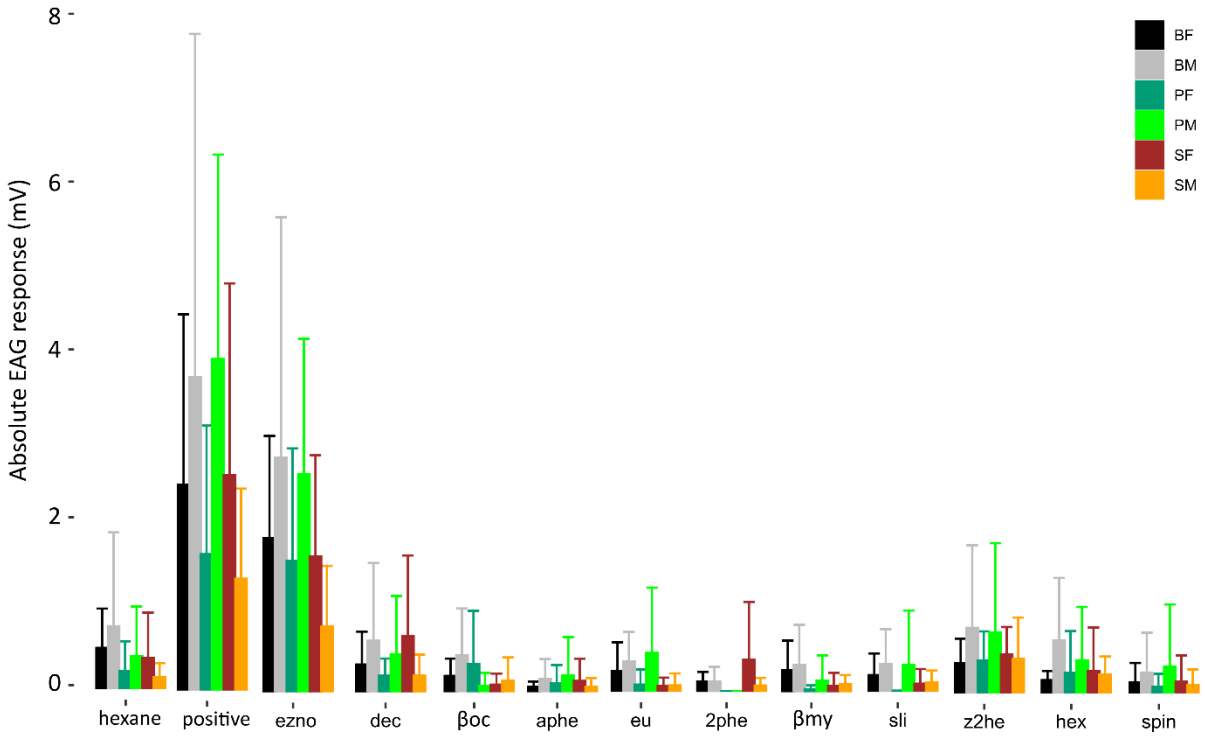
5.6 Supplementary Materials



Supplementary Figure 5.1 Antenna response after 10, 20, 30 and 40 seconds of recovery time when exposed to (*S*)-(-)-limonene (**A**), (*E*,*Z*)-2,6-nonadienal (**B**), and eucalyptol (**C**).



Supplementary Figure 5.2 Peak size of 11 test compounds detected by FID after 0 (**A**), 5 (**B**) and 10 (**C**) puffs. Chemical peaks: 1 = (*Z*)-2-hexen-1-ol, 2 = hexanal, 3 = (*1S*)-(-)-α-pinene, 4 = β-myrcene, 5 = α-phellandrene, 6 = eucalyptol, 7 = (*S*)-(-)-limonene, 8 = β-ocimene, 9 = 2-phenylethanol, 10 = (*E*,*Z*)-2,6-nonadienal, 11 = decanal.



Supplementarry Figure 5.3 Absolute electroantennogram (EAG) responses of New Zealand alpine grasshoppers to hexane and positive (β-caryophyllene) controls and 11 plant volatiles at concentrations of 0.1 mg/mL observed. Abbreviations: ezno = (*E,Z*)-2,6-nonadienal, dec = decanal, βoc = β-ocimene, αphe = α-phellandrene, eu = eucalyptol, 2phe = 2-phenylethanol, βmy = β-myrcene, sli = (*S*)-(-)-limonene, z2he = (*Z*)-2-hexen-1-ol, hex = hexanal, αpine = (*1S*)-(-)-α-pinene; BF = *Brachaspis nivalis* female; BM = *B. nivalis* male; PF = *Paprides nitidus* female; PM = *P. nitidus* male; SF = *Sigaus australis* female; SM = *S. australis* male.

Supplementary Table 5.1 Chemical compounds found in leaf extracts of *Celmisia spectabilis* from Central Plateau after 2 hours, 5 hours and 24 hours of hexane extraction.

Chemicals	2h	5h	24h
Terpenoids			
α-Pinene			+
Limonene		+	+
Green leaf volatiles			
Hexanal	+	+	+
(*E*)-3-Hexen-1-ol	+	+	+
(Z)-3-Hexen-1-ol	+	+	+
4,8-Dimethyl-1-nonanol			+
Nonanal			+
(*E,Z*)-2,6-Nonadienal			+
Ketone			

2-Heptanone	+

Supplementary Table 5.2 Chemical compounds found from a head-space collection of above ground foliage of three *Celmisia* species in Broken River Ski Area. Compounds in bold are the compounds used for electrophyisiological analysis.

Chemicals	***Celmisia lyallii***	***Celmisia spectabilis***	***Celmisia viscosa***
Terpenoids			
α-Pinene	+	+	+
β-Pinene	+	+	+
β-Myrcene			+
α-Phellandrene		+	+
Limonene	+	+	+
Eucalyptol	+	+	+
Ocimene		+	+
Linalool		+	
trans-α-Bergamotene			
Caryophyllene	+	+	+
α-Farnensene	+		
Green leaf volatiles			
(*E*)-2-Hexenal			+
Nonanal	+		+
Aromatic compounds			
o-Cymol	+	+	+

Supplementary Table 5.3 Chemical compounds found in leaf extracts of alpine plants in Ruahine Ranges. Compounds in bold are the compounds used for electrophyisiological analysis.

Chemicals	Grass	Dicot herbs	Shrubs		
	Chionochloa	*Celmisia spectabilis*	*Veronica odora*	*Dracophyllum*	*Macrolearia colensoi*
Terpenoids					
α-Pinene	+	+			+
Green leaf volatiles					
Hexanal	+	+	+	+	+
(*E*)-3-Hexen-1-ol	+	+	+	+	+
(*Z*)-3-Hexen-1-ol	+	+	+	+	+
(*Z*)-2-Hexen-1-ol	+		+		+
Octanol	+				+
4,8-Dimethyl-1-Nonanol	+	+	+		
(*Z,Z*)-3,6-Nonadienal					
Nonanal	+	+			+
(***E,Z*)-2,6-Nonadienal**	+				
cis-Geraniol			+		+

Supplementary Table 5.4 Chemical compounds found in leaf extracts of alpine plants in Waiouru Military Ground (Central Plateau). Compounds in bold are the compounds used for electrophyisiological analysis.

	Grass		Dicot herbs		Shrubs		
Chemicals	*Chionochloa*	*Poa colensoi*	*Anisotome aromatica*	*Celmisia spectabilis*	*Gaultheria crassa*	*Podocarpus nivalis*	*Veronica odora*
Terpenoids							
α-Pinene			+	+		+	+
Camphene			+				
β-Myrcene			+				
Limonene			+				+
Eucalyptol							+
Copaene							+
Green leaf volatiles							
Hexanal	+	+			+	+	+
(*E*)-3-Hexen-1-ol	+				+		+
(*Z*)-3-Hexen-1-ol	+				+	+	+
Octanol							+
4,8-Dimethyl-1-nonanol			+			+	
2-Hexyl-1-octanol							+

Supplementary Table 5.5 Chemical compounds found in leaf extracts of alpine plants in Tongariro National Park.

Chemicals	Grass	Dicot herbs		Shrubs
	Chionochloa	*Gentianella corymbifera*	*Celmisia spectabilis*	*Veronica odora*
Terpenoids				
α-Pinene		+		
Green leaf volatiles				
Hexanal	+	+	+	
(*E*)-3-Hexen-1-ol	+			+
(*Z*)-3-Hexen-1-ol	+			+
4,8-Dimethyl-1-nonanol		+	+	+
(*Z,Z*)-3,6-Nonadienal				
Nonanal			+	

Supplementary Table 5.6 Average normalized electroantennogram response (±SD) to 11 test stimuli at 100mg/mL of hexane in males and females of three New Zealand alpine grasshopper species. Normalized responses were calculated by dividing absolute EAG response in relation to response to positive control.

		Terpenoids						Green leaf volatiles				Aromatic compounds
Species	**sex**	β-Ocimene	α-Phellandrenephe	Eucalyptol	β -Myrcene	(*S*)-(-)-Limonene	(1*S*)-(-)-α-Pinene	(*E*,*Z*)-2,6-Nonadienal	Decanal	(*Z*)-2-Hexen-1-ol	Hexanal	2-Phenylethanol
B. nivalis	F	1.44 (±0.20)	2.04 (±0.30)	0.86 (±0.33)	1.36 (±0.34)	2.06 (±0.25)	0.84 (±0.27)	4.25 (±1.28)	3.60 (±0.79)	6.81 (±2.32)	3.03 (±2.84)	4.17 (±0.80)
	M	1.85 (±1.03)	2.43 (±0.86)	1.38 (±0.75)	1.94 (±0.95)	2.13 (±1.05)	1.05 (±0.34)	5.33 (±2.85)	3.52 (±1.63)	8.28 (±4.48)	3.51 (±3.50)	4.51 (±2.06)
P. nitidus	F	2.31 (±0.36)	2.95 (±0.40)	1.51 (±0.22)	2.46 (±0.33)	2.87 (±0.32)	1.42 (±0.23)	5.99 (±0.77)	4.25 (±0.92)	9.35 (±0.56)	4.93 (±2.23)	4.98 (±0.30)
	M	2.14 (±0.54)	2.72 (±0.46)	1.39 (±0.50)	1.97 (±0.62)	2.48 (±0.56)	1.36 (±0.36)	5.44 (±1.23)	3.88 (±0.76)	8.62 (±1.77)	4.60 (±3.41)	4.9 (±1.08)
S. australis	F	3.234 (±2.14)	3.644 (±2.29)	1.56 (±1.45)	3.134 (±2.83)	3.19 (±1.36)	1.96 (±1.43)	7.18 (±4.57)	4.59 (±2.01)	12.60 (±12.41)	4.31 (±4.55)	6.43 (±3.73)
	M	1.61 (±1.04)	2.13 (±1.04)	1.30 (±0.64)	1.72 (±0.93)	1.52 (±1.05)	1.33 (±0.75)	4.03 (±2.0)	2.42 (±1.19)	6.72 (±3.29)	2.05 (±1.08)	3.69 (±1.73)

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What percentage of the manuscript/published work was contributed by the student?	80%
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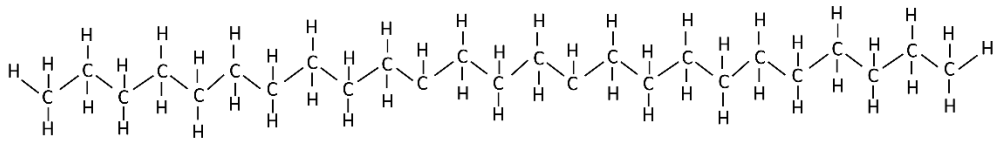
Chapter 6

Sexual Communication in the New Zealand Alpine Grasshoppers

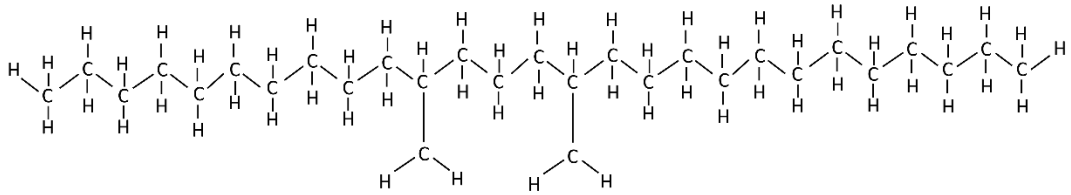
6.1 Introduction

Although the majority of Caelifera (grasshoppers, locusts, and their allies) can detect sound, acoustic communication is uncommon in this group of insects (Song et al. 2020). Instead, most Caelifera use an array of complex chemical signals for communication (Chapter 2). Insect-derived chemicals include cuticular hydrocarbons (CHCs) and volatiles (i.e., pheromones). All insect cuticles contain hydrocarbons made of tight associations between hydrophobic hydrocarbons derived from fatty acids, which are important for limiting water-loss through the cuticle (Blomquist et al. 2018; Menzel et al. 2019). CHCs typically comprise 20 to 40+ carbon atoms comprising alkanes, sometimes including double bond(s) (e.g., alkenes and alkadienes) and/or methyl branches (Gibbs and Rajpurohit 2010; Menzel et al. 2017a; Blomquist et al. 2018) (Figure 6.1). In insects, these compounds vary in the carbon chain length, and position and number of methyl branches and/or double bonds.

Pentacosane



11,15-Dimethylheptacosane



(Z,Z)-6,9-Heptacosadiene

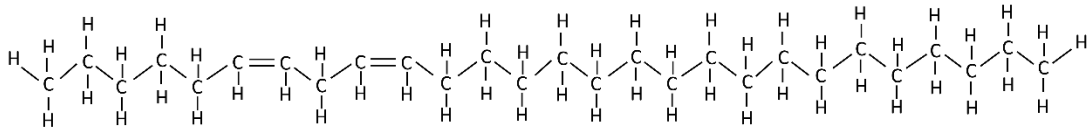


Figure 6.1 Bond line diagrams of carbon chain types found in cuticular hydrocarbons (CHCs) of insects. Example of n-alkane (pentacosane); methyl branched alkane with two methyl groups (13,15-dimethylheptacosane); alkadiene i.e., unsaturated alkane with two cis double bonds ((Z,Z)-6,9-heptacosadiene).

Despite their function in limiting water loss, the exact characteristics and components of CHC profile seem to be specific to a particular taxonomic group (Blomquist et al. 2018; Holze et al. 2021). Representatives of five subfamilies of grasshoppers (Cyrtacanthacridinae, Oedipodinae, Gomphocerinae, Melanoplinae, and Acrididae) have been examined (Table 6.1) and all their CHC have *n*-alkane chains or methyl-branched (mono-, di- and tri-) alkanes with a typical backbone carbon chain length between C22 to C37 (Lockey 1976; Lockey and Oraha 1990; Chapman et al. 1995, 2000; Hooper et al. 1996; Sutton et al. 1996; Finck et al. 2016a). In contrast, both saturated (*n*-alkanes and branched alkanes) and unsaturated (alkenes, alkadienes) hydrocarbons are found in some crickets (Thomas and Simmons 2008; Tyler et al. 2015), ants

(Guillem et al. 2016; Sprenger et al. 2018; Menzel et al. 2019; Walsh et al. 2020), bees, wasps (Hadley et al. 1981; Kather and Martin 2015; da Silva et al. 2021; Maihoff et al. 2023), flies (Howard et al. 2003; Fedina et al. 2012; Snellings et al. 2018; Moore et al. 2021; Dutta et al. 2022) and beetles (Page et al. 1990a, b; Botella-Cruz et al. 2017).

CHC profiles are influenced by environmental factors including climate (Neems and Butlin 1995; Rourke and Gibbs 1999; Menzel et al. 2017a, c; Sprenger et al. 2018) and diet (Fedina et al. 2012; Schwander et al. 2013; Otte et al. 2015). Temperature and humidity are especially important factors in shaping insect CHC profiles as it affects insect water balance, and at the same carbon chain length, water-proofing qualities are scaled in the order of *n*-alkanes > methyl-branched alkanes > unsaturated alkanes (Menzel et al. 2017b; Sprenger et al. 2018). A higher proportion of *n*-alkanes to branched alkanes have been observed in locust *Schistocerca shoshone* from populations with higher temperatures (5–10 °C difference; Chapman et al. 1995). These insects retained the same CHC profile even when they were reared in the laboratory conditions from the egg to the adult stage indicating their CHC profiles are conserved. However, CHC profiles can also be plastic in other insects. For example, a higher proportion of *n*-alkanes to branched and unsaturated alkanes have been observed within two to three weeks in the ants maintained in drier conditions (40–60 % relative humidity) compared to humid (90 % RH) (Menzel et al. 2017c) or in higher temperature (28 °C) compared to low (12–20 °C) (Sprenger et al. 2018).

Table 6.1 Presence/absence (+/-) of chemical classes (n-alkanes, methyl-branched alkanes, alkenes and alkadienes) in the cuticular hydrocarbons of different insects. Abbreviations: mb = methyl-branched, W = wild caught, L = lab-reared. W* indicates wild-caught but maintained in captivity for a certain time; +/- indicates the presence or absence of a chemical class depends on the species or localities studied.

	Saturated		Unsaturated		C chain	Wild/Lab	Country	Reference
	n-Alkanes	mb-alkanes	Alkenes	Alkadienes	length			
Flies								
Drosophila suzuki	+	+	+	+	14-34	L	Belgium	Snellings et al. 2018
Drosophila melanogaster	+	+	+	+	17-30	L	India	Dutta et al. 2022
Drosophila birchii	+	+	+	+	20-33	W	Australia	Howard et al. 2003
Drosophila serrata	+	+	+	+	24-31	W	Australia	
Sarcophaga spp.	+	+	+/-	+/-	21-35	W	UK	Moore et al. 2021
Wasps and Bees								
Bombus spp.	+	+/-	+	+/-	21-33	W	Germany	Maihoff et al. 2023
Apis mellifera	+	+	+	+	18-33	L	US	Vernier et al. 2019
Vespula germanica	+	+	+	-	11-39	W	Belgium	da Silva et al. 2021
Bembix pruinosa	+	+	+	-	24-37	W	US	Hadley et al. 1981
Ants								
Myrmica spp.	+	+	+/-	+/-	21-39	W	Finland, Spain, UK, Greece	Guillem et al. 2016
Monomorium pharaonis	+	+	+	+	25-33	L	?	Walsh et al. 2020
Beetles								
Dendroctonus ponderosae	+	+	+	-	22-38	W	US	Page et al. 1990a
Conophthorus spp.	+	+	+	+	21-45	W	US, Canada	Page et al. 1990b
Nebrioporus baeticus	+	+	+	-	16-31	W*	Spain	Botella-Cruz et al. 2017
Enochrus jesusarribasi	+	+	+	-	16-36	W*	Spain	
Grasshoppers								
Locusta migratoria migratoriodes	+	+	-	-	14-37	L	UK	Lockey and Oraha 1990
Schistocerca gregaria	+	+	-	-	14-36	L	UK	
Chorthippus biguttulus	+	+	-	-	25-39	W*	Germany	Finck et al. 2016a
Chorthippus mollis	+	+	-	-	25-37	W*	Germany	
Halmenus robustus	+	+	-	-	23-37	W	Ecuador	Chapman et al. 2000
Schistocerca gregaria	+	+	-	-	23-37	W	Ecuador	
Schistocerca melanocera	+	+	-	-	23-37	W	Ecuador	
Chortoicetes terminifera	+	+	-	-	23-47	W	Australia	Hooper et al. 1996
Crickets and allies								
Gryllotalpa spp.	+	+	-	-	20-29		Israel	Broza et al. 1998
Hemideina maori	+	+	-	-		W	New Zealand	Hadley et al. 1988
Teleogryllus oceanicus	+	+	+	+	31-35	L	Australia	Thomas & Simmons 2008
Gryllus spp.	+	+	+	+	25-31	L	Spain	Tyler et al. 2015

Genetic understanding of CHC synthesis is limited and focused on model organisms like fruit flies *Drosophila* species or locust *Locusta migratoria* (Holze et al. 2021). CHC profiles are controlled by a variety of catalysts involved in the biosynthesis of a particular CHC class (Blomquist et al. 2018; Holze et al. 2021). For example, fatty acid synthase (FAS) is considered to be involved in the synthesis of methyl-branched alkanes (Finck et al. 2016a; Holze et al. 2021) and knockdown of one of the three fatty acid synthases (*LmFAS3*) found in *L. migratoria* by RNA interference reduced the methyl-branched content of their CHC profiles (Yang et al. 2020). It is yet to be known whether environmental fluctuations affect the expression levels of particular genes involved in the biosynthesis of CHCs (Holze et al. 2021). As only *n*-alkanes and methyl-branched alkanes are present in acridid grasshopper species studied so far (Table 6.1), it is possible that these CHC compounds are conserved in this group.

Alpine insects are subject to higher risk of desiccation compared to low elevation species due to high wind speed and higher intensity of solar radiation and have therefore evolved traits that are effective in preventing water loss (Hodkinson 2005; King and Sinclair 2015). In the New Zealand Orthoptera *Hemideina* wētā, water loss through cuticle and respiration was twice the rate in lowland species (*H. crassidens*, *H. thoracica* and *H. femorata*) compared to montane species (*H. maori* and *H. ricta*) when exposed to dry air, showing the montane species are better at preventing water loss than their low elevation relatives (King and Sinclair 2015).

Insect cuticular hydrocarbons also serve as communication signals that are important in recognition of nestmates (Brandstaetter et al. 2008), populations (Chapman et al. 1995; Neems and Butlin 1995; Tregenza et al. 2000; Menzel et al. 2017a), species and sex (Lockey 1976; Hadley et al. 1981; Chapman et al. 2000; Zhang et al. 2011; Schwander et al. 2013; Finck et al.

2016b, a). For example, two sympatric *Chorthippus* grasshopper species recognize their own species by hydrocarbons with different branching patterns (Finck et al. 2016a, b).

Insects can receive chemical signals by touching (gustation) or without making direct contact (olfaction). CHCs are often referred to as contact-chemicals or short-range-olfactory in contrast to volatiles which are considered as long-distance-olfactory signalling (Chapter 2). This is due to the higher molecular weight and lower volatility of hydrocarbons compared to volatiles. Although studies regarding sexual communication in grasshoppers are limited, they use both volatiles and CHCs to communicate. Flighted species that can disperse long distances (e.g., locusts, >100 km a day) rely on long-distance volatiles while species that do not travel long distances (e.g., *Chorthippus* grasshoppers, <3 m: Bailey et al. 2003; Tim and Hill 2004; Weyer et al. 2012; Ortego et al. 2021) are reliant more on CHCs (Chapter 2). New Zealand alpine grasshoppers are flightless and dispersal distance per day range between 0.3–3 m in males and 0.06 –1 m in females (White 1974b); are therefore likely to rely on CHC for intraspecific communication.

In this chapter, chemical profiles of males and females of the three South Island alpine grasshopper species *Brachaspis nivalis, Sigaus australis* and *Paprides nitidus* and one North Island species *Sigaus piliferus*, are measured and compared. These grasshoppers are exposed to dry mountain climates, where the temperature can reach >30 °C and relative humidity can drop to 30 % on summer days (information derived from data logger February–March 2021). In addition, North Island mountains are generally hotter and drier than Southern Alp habitat (National Institute of Water and Atmospheric Research (NIWA) 2022) so *S. piliferus* may have a higher risk of desiccation than South Island species. If so, it may display CHC composition more

suitable for desiccation resistance. As New Zealand alpine grasshoppers do not actively sing to attract mates, I also predict that sexual differences will exist that allow individuals to recognise potential mates. The distribution of sensilla on the antennae of males and females is very similar (Chapter 4) and therefore it is possible that both males and females are using CHC clues to recognise potential mates. Sympatric South Island species are expected to each have different chemical profiles that facilitate successful identification of individuals of their own species and matching sex. In contrast, an allopatric species from the same insect radiation could have an overlapping CHC composition with southern species as there is less selection pressure to differentiate species.

6.2 Methods

Insects

Adult grasshoppers were collected during their active summer season with approval from the operators of the ski field and military ground, and Department of Conservation (authorization number: 97397-FLO). *Brachaspis nivalis*, *Sigaus australis* and *Paprides nitidus* were collected at Hamilton Peak in the Craigieburn Range, South Island (−43.125929, 171.687432). *Sigaus piliferus* adults were collected at two sites of the North Island; Waiouru Military Camp Site 3, Central Plateau, (−39.308660, 175.746340) and Taranaki Falls Track, Tongariro National Park (−39.20219, 175.560294). Live specimens were transported to the laboratory at Massey University (Palmerston North, New Zealand), and kept at 4^{o}C with their food plants (*Celmisia spectabilis* and *C. lyallii* for South Island species, *Coriaria arborea* and *Macrolearia colensoi* for North Island species) with natural light prior to CHC collection.

Cuticular hydrocarbon extraction and analysis

Seven adults of each sex and species were subject to chemical analysis. Grasshoppers were frozen for 1 day at −20 °C and each submerged in 1mL of hexane containing 10 ng/µL of nonyl acetate as an internal standard (IS) for ten minutes. Blank hexane with the internal standard was treated the same way to provide a control and to check for contamination.

Samples were analysed in a gas chromatograph-mass spectrometer (GC-MS-QP2010, Shimadzu Corporation, Kyoto, Japan) using a TG-5MS (30 m length, 0.25 mm diameter, 0.25 µm thickness, Thermo Fisher Scientific). One microliter of cuticular extract was injected in a

splitless mode at a temperature of 300 °C with helium gas at a constant flow rate of 2.25 mL/min. The temperature was programmed as followed: 60 °C for 1 minute, then increased to 200 °C at a rate of 20 °C /min, and the final temperature was 280 °C held for 5 minutes (Golian et al. 2022). Peaks were scanned for mass to charge ratio (*m/z*) of 41 to 700 for diagnostic ions.

Alkanes were identified by comparing retention times and mass spectra to those in the NIST (National Institute of Standards and Technology) library 2005 and on the comparison of their retention times with reference C7 to C40 alkanes (No. 49452-U, Supelco). Branching positions of methyl alkanes were determined by examination of diagnostic ion fragments (Table 6.2). Due to the low solubility of oleamide in hexane, 5 mg of the standard was first dissolved in 1 mL of 96 % ethanol and mixed with hexane (Zhang et al. 2011). The quantity of each compound was calculated by comparing the peak area of each detected compound to that of the internal standard. Proportions of the hydrocarbon classes, *n*-alkanes, monomethyl-alkanes and dimethyl-alkanes, were calculated to compare hydrocarbon composition with other insect species.

Statistical analysis

Statistical analyses were performed and graphs were produced in the R statistics environment (R Core Team 2023) using the software platform RStudio 4.0.3 (Boston, MA, USA). A distance matrix of pairwise Bray-Curtis dissimilarity indices was built for quantitative comparisons of the CHC profiles among species and between sexes of the alpine grasshoppers. A non-metric multidimensional scaling (NMDS) was used to visualize similarities among the groups using R package “vegan” (Oksanen et al. 2022). Similarity percentage (SIMPER) was used to identify the compounds that accounted for >70 % dissimilarities between the groups (Effah et al. 2020).

Statistical normality was tested using a Kolmogorov-Smirnov test and a student T-test was performed to test the quantitative differences between males and females for those compounds contributing >70 % dissimilarities. The linear model followed by a Tukey's pairwise test were used to test quantitative differences between species and a student T-test was used to test quantitative difference between sexes.

Additional preliminary analyses

In addition to the CHC analysis, head-space collection of volatiles from grasshoppers and electroantennogram (EAG) analysis were performed. Insect-derived volatiles were collected at Broken River Ski Field in February 2021. Full-body headspace collections (Nakano et al. 2019) from males and females from each species were sampled (n= 4 for each sex per species) for three timeframes over nine hours (07:00–10:00, 11:00–14:00, 16:00–19:00) with ambient light and temperature. These time frames were used to observe changes in chemical profiles over time (if any) when the grasshoppers are active.

Samples were analysed using a Gas Chromatograph – Mass Spectrometer (GCMS-QP2010, Shimadzu Corporation, Kyoto, Japan), using a DB-5 capillary column (30 m× 0.25 mm ID, J & W Scientific, Folsom, CA, USA). The samples were injected in split mode, and the temperature was programmed for 3min at 50 °C then increased to 95 °C at 5 °C/min, 145^{o}C at 15 °C/min, 180 °C at 10 °C/min, and finally 200 °C at 10 °C/min (23.83 minutes total). Compounds were identified by comparing retention times and mass spectra to those in the NIST (National Institute of Standards and Technology) Library 2005.

Electroantennogram (EAG) analysis was performed with synthetic oleamide (No. O2136, Sigma-Aldrich) on *B. nivalis*, *S. australis* and *P. nitidus* (February 2023). Ten microlitres of oleamide dissolved in 95 % ethanol was laced on piece of filter paper, and the ethanol allowed to evaporate for 10 seconds in a fume hood before being loaded into a Pasteur pipette (Sigma-Aldrich). A range of oleamide concentrations were used: 0.1 mg/mL, 1mg/mL and 5.99 mg/mL EAG responses to stimuli were recorded from lower to higher doses, and a blank air puff and solvent control (95 % ethanol) were used at the beginning of the experiment to compare the response between these controls and the test stimuli (oleamide). Due to the limited number of available grasshoppers this analysis was performed with one or two individuals per sex of each species (repeated two to three times per individual). Antenna preparation and EAG setups were as described in Chapter 5.

6.3 Results

Cuticular hydrocarbon analysis

Cuticular extracts of the four New Zealand alpine grasshopper species comprised 25 different peaks (Figure 6.2). These peaks represented fatty amides (oleamide and octadecanamide), *n*-alkanes and methyl-branched alkanes (monomethyl- and dimethyl-alkanes) with carbon chains ranging from C25 to C37 (Table 6.2). Methyl-branched alkanes were more abundant than *n*-alkanes in *P. nitidus*, while *n*-alkanes were similar or in greater abundance than methyl-branched alkanes in *B. nivalis*, *S. australis*, and *S. piliferus* (Figure 6.3; Supplementary Table 6.1). *n*-C27 and *n*-C29 were the dominant compounds in all species, together comprising almost 30 % or more of the total hydrocarbon (peaks 6 & 7 in Figure 6.2; Supplementary Table 6.1). Mass

spectra indicated that three peaks (peaks 1–3 in Figure 6.2) were derived from oleamide (Supplementary Figure 6.1) and could potentially represent isomers.

Table 6.2 Hydrocarbons detected in cuticular extracts from New Zealand grasshoppers ordered by GC retention time. Peak number (corresponding to number in Figure 6.2), retention time (RT), compound identity and diagnostic ions and Kovats index. First (and second) number(s) of a compound indicate(s) the position of

Peak number	RT	Compound	Carbon chain length	Diagnostic ions m/z			Kovats Index
				Base peak	Specific ion	Molecular peak	
1	19.191	Oleamide1	18	59		281	2364
2	19.3	Oleamide2	18	59		281	2368
3	19.408	Oleamide3	18	59		281	2369
4	19.684	Octadecanamide	19	59		283	2387
5	22.21	*n*-C25	25	57		352	2500
6	26.845	*n*-C27	27	57		380	2700
7	31.36	*n*-C29	29	57		408/409	2900
8	35.7	*n*-C31	31	57		436/437	3100
9	41.043	18-MeC33	33	57	267	478	3354
10	41.16-17	15-MeC33	33	57	225	478	3360
11	41.5	15,18-diMeC33	33	57	225, 323 or 168, 239	492	3377
12	41.8	7,11-diMeC33	33	57	112, 183	492	3391
13	42.05	*n*-C34	34	57	478/479	478/479	3400
14	42.263	18-MeC34	34	57	183	492	3413
15	42.565	10-MeC34	34	57	155	492	3428
16	43.1	13,y-diMeC34	34	57	196	506/507	3454
17	44.5	13-MeC35	35	57	196	506/507	3523
18	45.2	11-MeC35	35	57	168	506/507	3554
19	45.723	13 or 11,y-diMeC35	35	57	196 or 168/169	520/521	3582
20	45.8-9	7,18-diMeC35	35	57	112/113, 280	520/521	3587
21	46.1	*n*-C36	36	57		506	3600
22	46.5	19 or 17-diMeC36	36	57	280/281 or 211	534	3699
23	48.8	13-MeC37	37	57	196	534	3732
24	49.27	11-MeC37	37	57	168	534	3756
25	49.7	11,19-diMeC37	37	57	168/169, 294/295	548	3775

methyl-branch(es).

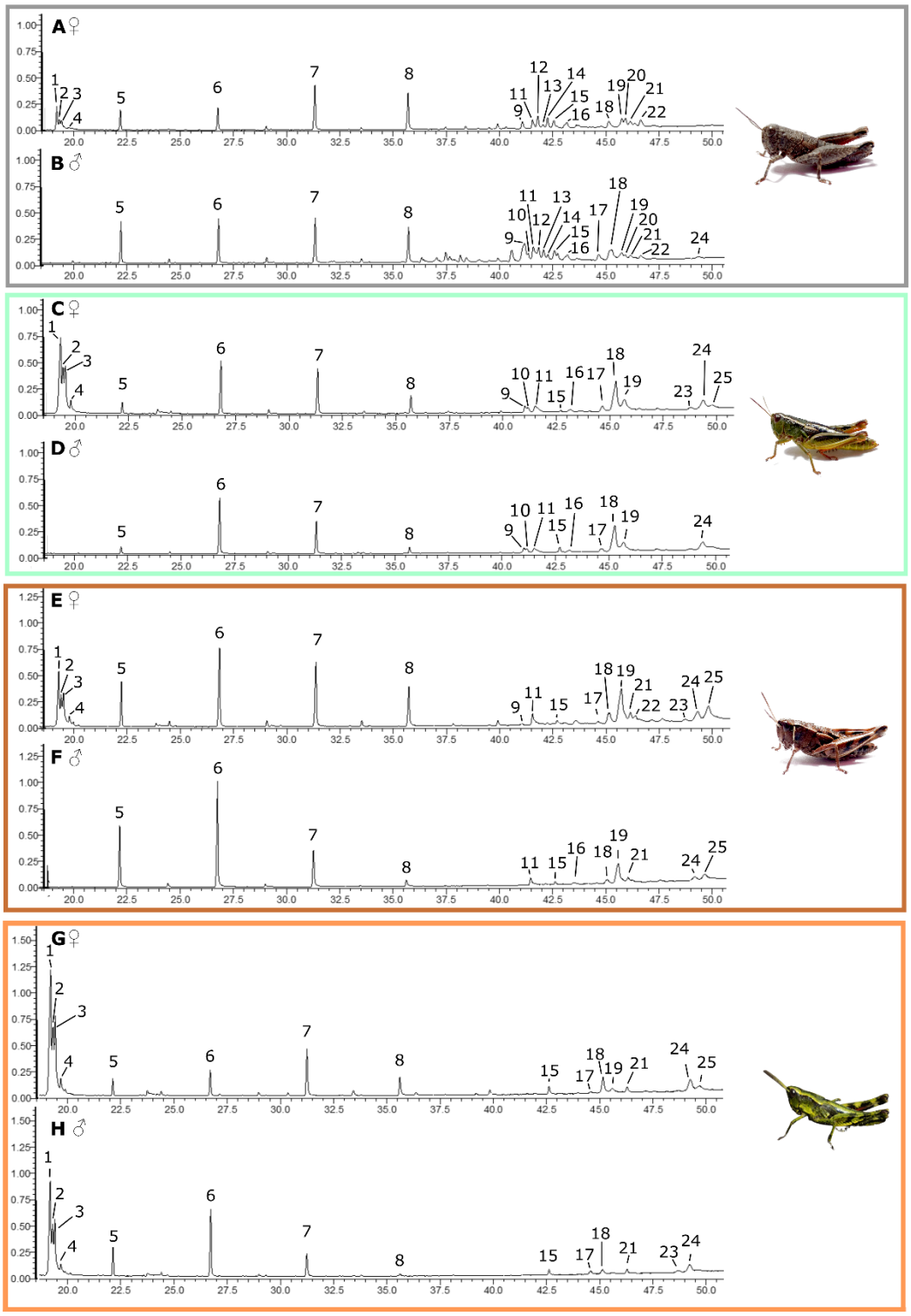


Figure 6.2 Examples of gas chromatograms of hexane extracted cuticular hydrocarbons from representative individuals of four New Zealand alpine grasshopper species: *Brachaspis nivalis* female (**A**) and male (**B**); *Paprides nitidus* female (**C**) and male (**D**); *Sigaus australis* female (**E**) and male; *Sigaus piliferus* female (**G**)

and male (**H**). X-axis: retention time (in minutes), y-axis: relative abundance. Numbers Peak numbers correspond to compounds in Table 6.2.

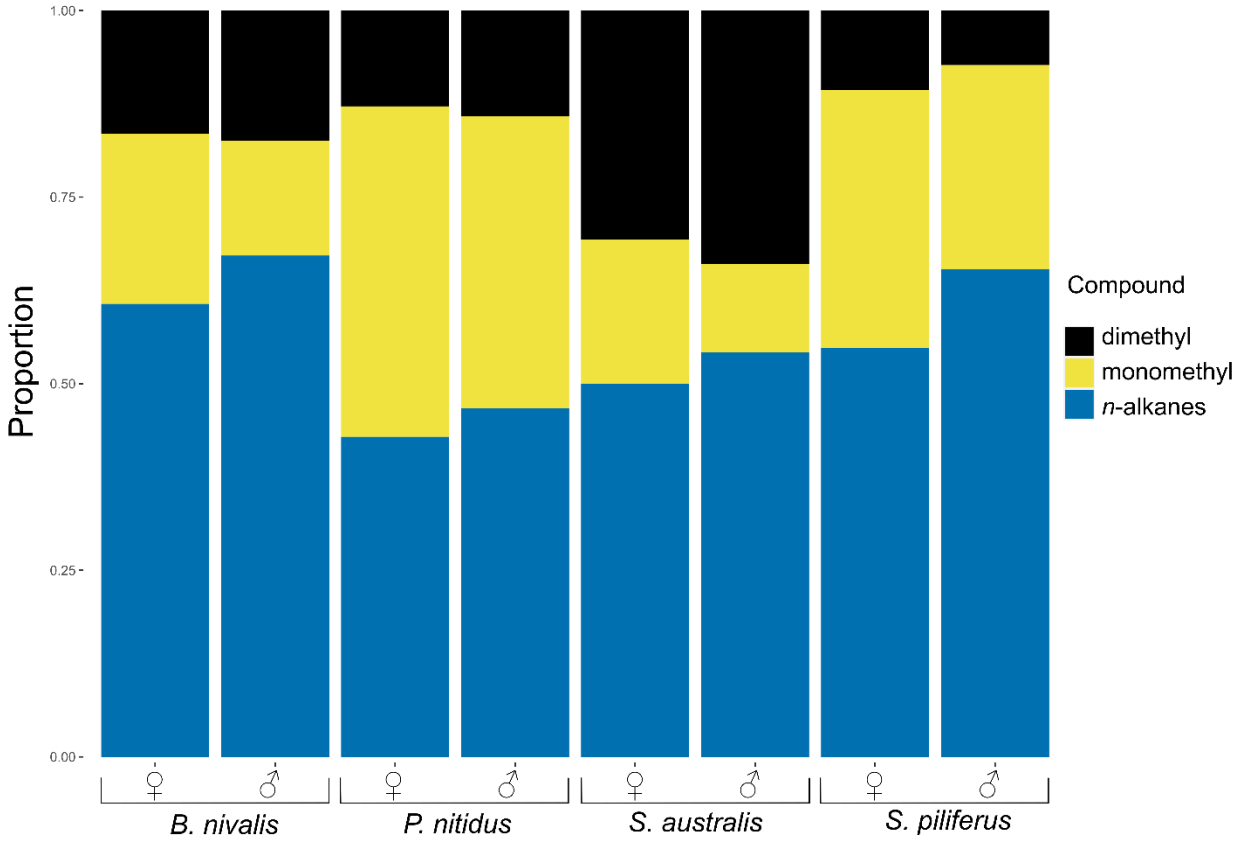


Figure 6.3 Relative composition of major hydrocarbon groups, dimethyl-alkanes, monomethyl-alkanes and *n*-alkanes, extracted from the cuticles of four species of New Zealand alpine grasshopper: *Brachaspis nivalis*, *Paprides nitidus*, *Sigaus australis* and *Sigaus piliferus*.

A two-dimensional nMDS ordination (stress= 0.138) showed a clear separation of CHC profiles from four species (Figure 6.4). The CHC composition of South Island *P. nitidus* was more similar to that of North Island *S. piliferus* than to South Island *B. nivalis*. CHC profiles of males and females of North Island *S. piliferus* showed an overlap, while South Island species showed a clear separation between sexes and females of *P. nitidus* and *S. australis* showed an overlap.

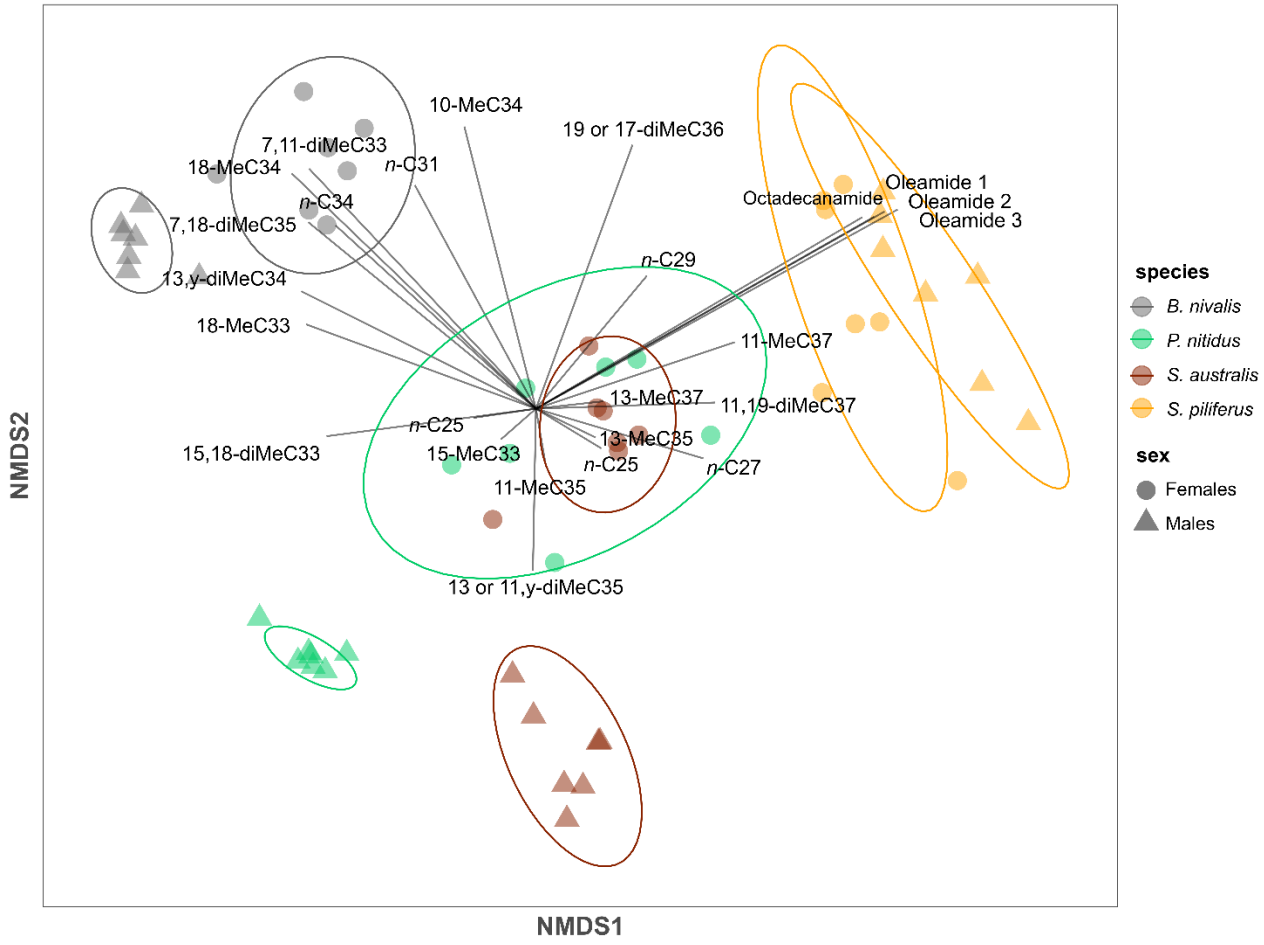


Figure 6.4 Cuticular hydrocarbon (CHC) profiles of males and females of four New Zealand alpine grasshoppers visualized using non-metric multidimensional scaling (NMDS) ordination based on a Bray-Curtis dissimilarity matrix. The ellipses show the 95% confidence areas around the centroids for each species and sex. North Island grasshopper: *Sigaus piliferus*; South Island grasshoppers: *Brachaspis nivalis*, *Paprides nitidus*, and *Sigaus australis.*

Percentage similarity (SIMPER) revealed oleamide 1 and 3, *n*-C27, *n*-C29, *n*-C31, 11-MeC35, 13 or 11,y-diMeC35, and 11-MeC37 contributed to separation between species, and oleamide 1 and 3 were responsible for separation between sexes in all species (Figure 6.5). In addition, *n*-C25 contributed separation between sexes of *B. nivalis* and *P. nitidus* (Figure 6.5C) and 11-MeC37 contributed separation between sexes of *S. piliferus* (Figure 6.5I). Many of these

compounds were emitted significantly more by *S. piliferus* than other species (Figure 6.5A, B, D, E, I), and oleamide 1 and 3 accounted for almost 40% of separation of *S. piliferus* from other species according to the SIMPER analysis. The species *B. nivalis* can be distinguished from the other species by elevated levels of *n*-C31 and was indicated as responsible for the separation of *B. nivalis* from other species and it was significantly more abundant in *B. nivalis* than in other species (Figure 6.5F). Although they did not account for large (70 %) differences in SIMPER analysis, the compounds 7,11-diMeC33, *n*-C34, 18-MeC34, 10-MeC34, and 13,y-diMeC35 were emitted significantly more by *B. nivalis* than other species (peaks 12–16 in Figure 6.2). The hydrocarbons 11-MeC35 in *P. nitidus* and 13 or 11, y-diMeC35 in *S. australis* were significantly more abundant than in other species (Figure 6.5G & H).

Between sexes, oleamide 1–3 (Figure 6.5A & B; peaks 1–3 in Figure 6.2) and octadecanamide (peak 4 in Figure 6.2) were emitted only by females of *B. nivalis*, *S. australis* and *P. nitidus*, and females of *P. nitidus* and *B. nivalis* emitted *n*-C25 significantly more than in conspecific females (Figure 6.5C). In contrast, both males and females of *S. piliferus* emitted oleamide 1–3 (peaks 1–3 in Figure 6.2) and females emitted oleamide significantly more than males (Figure 6.5A & B).

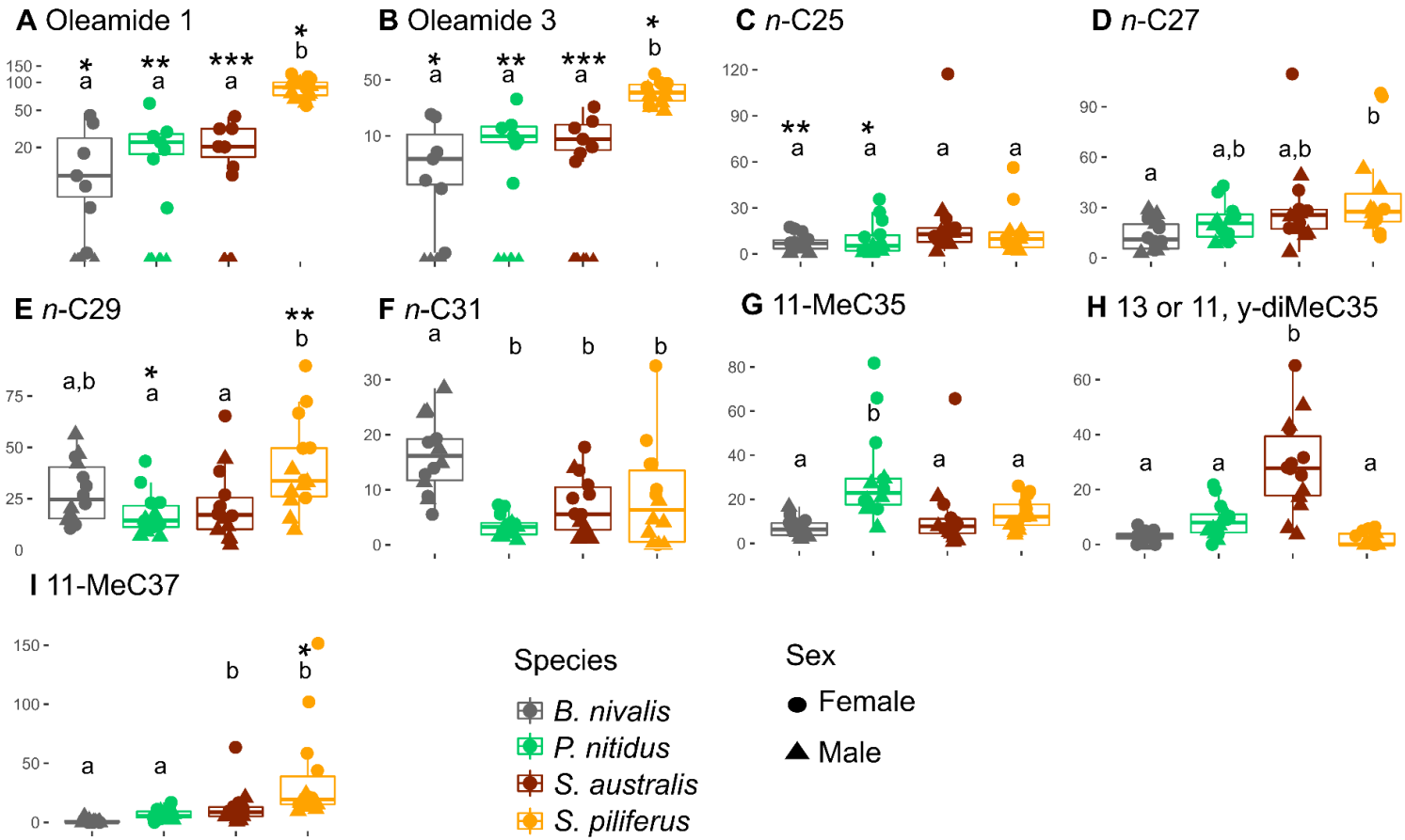


Figure 6.5 Quantities of cuticular hydrocarbons that accounted for >70% dissimilarities between species and sexes of four New Zealand alpine grasshoppers, *Brachaspis nivalis*, *Paprides nitidus*, *Sigaus australis* and *Sigaus piliferus*. The quantity of each compound was calculated by comparing the peak area of each detected compound to that of the internal standard. Colors represent species and shapes represent sexes. Different letters indicate significant differences among species using a linear model followed by a pair-wise post hoc Tukey honest significant test, and * indicates significant difference between sexes using a Student T-test. Oleamide quantities in **A** and **B** are on a log-transformed scale.

Other results

Full body head-space collection showed none of the compounds detected were uniformly emitted by grasshoppers. Compounds detected from some individuals include monoterpenes (α-pinene, β-pinene, β-myrcene, limonene) and green leaf volatiles ((*Z*)-3-hexen-1-ol-acetate; Figure 6.6).

Electroantennogram (EAG) responses (mV) to oleamide (dissolved in 95 % ethanol) were similar to those of 95 % ethanol in *B. nivalis*, *S. australis* and *P. nitidus* (Figure 6.7), indicating no olfactory response to oleamide. The intensity of EAG responses to oleamide at all dosages and 95 % ethanol were two to three times more than that of the air puff.

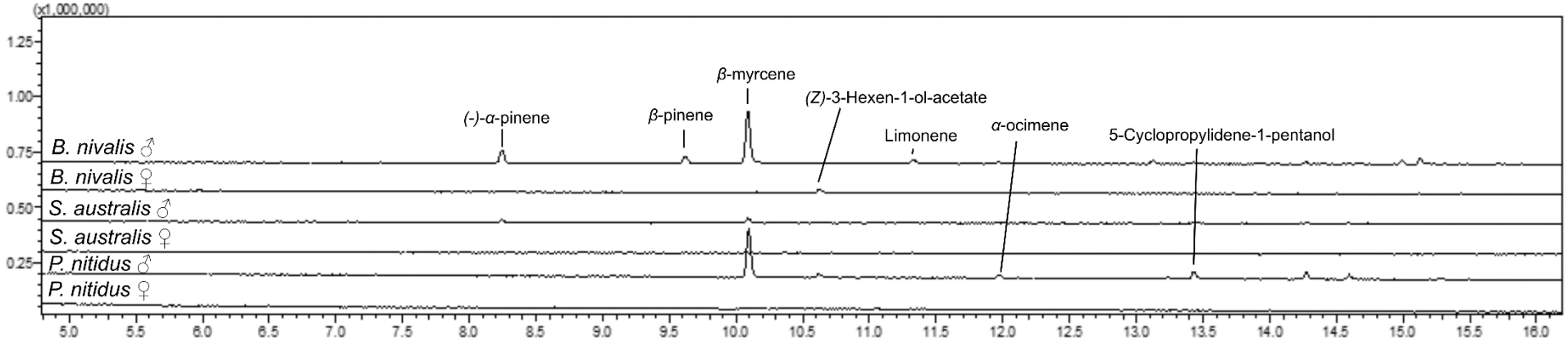


Figure 6.6 Gas chromatogram of volatiles found in three New Zealand alpine grasshopper species sampled by headspace collection. X-axis represents retention time and y-axis represents relative abundance of each compound.

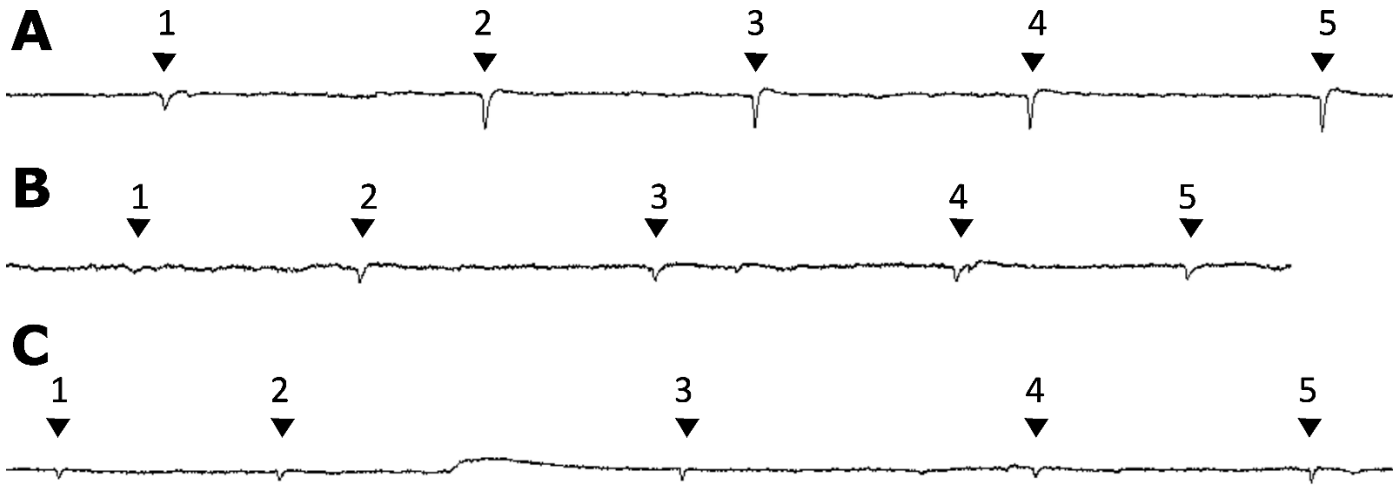


Figure 6.7 Electroantennogram responses to air puff (1), 95 % ethanol (2), oleamide at 0.1 mg/mL (3), 1 mg/mL (4) and 5.99 mg/mL (5) of 95 % ethanol in *Brachaspis nivalis* male (**A**), *Sigaus australis* female (**B**) and *Paprides nitidus* male (**C**).

6.4 Discussion

Type of compounds extracted from grasshopper cuticles

Fatty amides (oleamide and octadecanamide), *n*-alkanes and methyl-branched alkanes with chain lengths between C25 and C37 were present in cuticular extracts of four endemic New Zealand alpine grasshopper species (Table 6.2; Figure 6.2). Analysis of the head-space collection revealed that no volatile compounds were consistently emitted by the grasshoppers, but terpenoids and green leaf volatiles were detected from some individuals. These compounds are probably derived from plants as these compounds are also detected in grasshopper food plants (Chapter 5).

Oleamide was present in relatively high quantity in extractions from the female grasshopper cuticles. However, the low solubility of oleamide in hexane (Zhang et al. 2011) probably resulted in underestimation of its abundance. These amides have not previously been reported in Acrididae. As in other acridids (Lockey 1976; Lockey and Oraha 1990; Chapman et al. 1995, 2000; Hooper et al. 1996; Sutton et al. 1996; Finck et al. 2016a), no unsaturated hydrocarbons (having double bond(s)) were found in the New Zealand alpine grasshoppers examined. As in New the Zealand alpine wētā *Hemideina maori*, only *n*-alkanes and methyl-branched alkanes were found (Hadley et al. 1988). In contrast, both unsaturated and saturated hydrocarbons were found in an alpine bumble bee *Bombus* from North America (Hadley et al. 1981) and Germany (Maihoff et al. 2023) but their mixed occurrence is not restricted to alpine habitat as other hymenopterans in non-alpine habitat (e.g., ants: Sprenger et al. 2018; Menzel et al. 2019; bees, wasps: Hadley et al. 1981; Kather and Martin 2015; da Silva et al. 2021).

C27 and C29 *n*-alkanes were found to be abundant in all four of the New Zealand species examined, and have been reported in other grasshopper species studied including *Schistocerca americana*, *Schistocerca melanocera* and *Halmenus robustus* from Galapagos Islands (Chapman et al. 2000), *Schistocerca gregaria* and *Locusta migratoria* from UK (Lockey and Oraha 1990), *Schistocerca shoshone, Melanoplus sanguinipes* and *Melanoplus spretus* from US (Chapman et al. 1995; Sutton et al. 1996), and *Chorthippus biguttulus* and *Corthippus mollis* from Germany (Finck et al. 2016a). The presence of C27 and C29 *n*-alkanes and absence of unsaturated hydrocarbons appears to be the stable CHC profile of acridid grasshoppers.

Among species

Cuticular hydrocarbon (CHC) composition is to some degree plastic and changes according to climate conditions (Menzel et al. 2017c; Sprenger et al. 2018) and food plants (Otte et al. 2015), but the New Zealand alpine grasshoppers maintained species and sexual difference even though they were kept in captivity for more than a week (due to transportation). Fixed CHC composition was also observed in *S. shoshone* when they were raised from eggs to adults in a controlled temperature and humidity environment that were different from the populations they came from (Chapman et al. 1995).

In short-horned grasshoppers, the profile of CHC is known to differ between closely related sympatric such as *Chorthippus biguttulus* and *C. mollis* in which the position of the first methyl-branch was found to differ (Finck et al. 2016a). The relative proportions of *n*-alkanes and methyl-branched alkanes differed between the locust species *L. migratoria* and *S. gregaria* (Lockey 1976), and between *Schistocerca americana*, *Schistocerca melanocera* and *Halmenus*

robustus of the Galapagos Islands (Chapman et al. 2000). The four New Zealand species studied here differed in the relative abundance of hydrocarbons with different chain lengths and branching patterns. Many of the compounds, and in particular oleamide, were more abundant in samples from *S. piliferus* than the other species. *n*-C31 and methyl-branched C33 and C34 were abundant in *B. nivalis*. *Sigaus australis* and *P. nitidus* had similar CHC profiles but dimethyl-C35 in *S. australis* and 11MeC35 in *P. nitidus* were observed in higher quantities than in other species. This species-specific abundance of particular compounds may reflect their importance for conspecific recognition.

Due to their low volatility and high molecular weight, CHCs are usually considered to be relevant only for short-distance olfactory or contact signalling (Blomquist et al. 2018), and olfactory response to straight-chain hydrocarbons up to *n*-C41 was detected in *Harpegnathos saltator* ants using electrophysiological analysis (Ghaninia et al. 2017). In *Chorthippus mollis* and *C. biguttulus* grasshoppers, the abundance of dimethyl- and trimethyl-branched C33 to C39 alkanes differed between species and sexes (Finck et al. 2016a). In captive observations most males of these grasshopper species made physical contact when recognizing their own species, however a few (<10 %) appeared to detect these chemicals through olfaction at a range of 0.5–1 cm (Finck et al. 2016b). It is not yet known whether New Zealand alpine grasshoppers detect hydrocarbons through olfaction and/or contact chemoreception.

The proportion of *n*-alkanes to methyl-branched alkanes was >60 % in *B. nivalis* (67 % in males, 63 % in females) but lower in *S. australis* (54 % in males, 50 % in females) and *P. nitidus* (47 % in males, 43 % in females). This may reflect the more richly vegetated habitat of *S. australis* and

P. nitidus compared to the rock-dominated substrate of *B. nivalis* (Watson 1970; Koot 2018), where desiccation risk could be higher.

Between sexes

While males and females of South Island species *B. nivalis*, *S. australis* and *P. nitidus* showed a clear difference in their CHC profiles, a greater overlap between *S. piliferus* sexes was observed (Figure 6.4). In *B. nivalis*, *S. australis* and *P. nitidus* fatty acid amides were found only in the females whereas both male and female *S. piliferus* emitted oleamide and octadecanamide. Nevertheless, these compounds were significantly more abundant in female *S. piliferus* than in males. Therefore it is likely that these alpine grasshoppers use this information from CHC profiles for differentiating males from females and recognising potential mates.

Oleamide and octadecanamide have not previously been reported from grasshoppers but oleamide has been detected in some aquatic invertebrates including shrimps (Zhang et al. 2011), sea skaters (Petrakis et al. 2003), and sea squirts (Meenakshi et al. 2012). In *Lysmata boggessi* shrimps, oleamide is the major pheromone component in euhermaphrodite phase (having both male and female functions), eliciting sexual behaviours by males (Zhang et al. 2011). As oleamide has low solubility in water, it remains intact on an animal's body in the aquatic environment and functions as a contact pheromone in these shrimps (Zhang et al. 2011). In the terrestrial environment, oleamide occurs naturally in some plants (Jiang et al. 2015; Saranya et al. 2019) and Asian corn borer *Ostrinia furnacalis* show olfactory responses to oleamide extracted from maize (Jiang et al. 2015). In my preliminary experiment, oleamide did not elicit an electroantennogram response by males or females of *B. nivalis*, *S. australis* and *P. nitidus*

despite its abundance within their CHCs. This observation from a small sample suggests that these grasshoppers might not use olfaction to perceive oleamide, but direct contact (gustation) instead may be involved in the recognition of oleamide. It is also possible that chemical cues are combined with visual and auditory information seen in other grasshopper species (e.g., *Angaracris barabensis*: Chen and Kang 2000; *Chorthippus parallelus parallelus* and *C. p. erythropus*: Ritchie 1990). Further studies involving behavioural, electrophysiological, auditory and visual analyses are required to fully understand the sexual communication systems in New Zealand grasshoppers, but the evidence presented here demonstrates that CHC profiles would allow discrimination of species and sex of sympatric alpine grasshoppers.

6.5 References

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6.6 Supplementary Materials

Supplementary Figure 1 Mass spectra of oleamide 1-3 and octadecanamide.

Supplementary Table 1 Proportion (in %) of hydrocarbons found in New Zealand alpine grasshoppers.

species	sex	*n*-C25	*n*-C27	*n*-C29	*n*-C31	18-MeC33	15-MeC33	15,18-diMeC33	7,11-diMeC33	*n*-C34	18-MeC34	10-MeC34	13,y-diMeC34	13-MeC35	11-MeC35	13 or 11,y-diMeC35	7,18-diMeC35	*n*-C36	19 or 17-diMeC36	13-MeC37	11-MeC37	11,19-diMeC37
B. nivalis	**F**	10.4 (±2.4)	13.0 (±2.6)	21.5 (±4.4)	12.2 (±3.1)	3.5 (±3.1)	3.1 (±5.6)	4.6 (±2.2)	4.4 (±1.1)	1.4 (±1.0)	2.4 (±1.1)	3.0 (±1.5)	2.9 (±1.4)	1.6 (±1.2)	6.9 (±2.5)	2.1 (±1.8)	1.7 (±1.0)	1.5 (±0.6)	1.6 (±0.5)	1.3 (±1.8)	0.8 (±1.0)	0.1 (±0.2)
	M	3.9 (±1.8)	13.0 (±4.9)	28.8 (±5.3)	18.9 (±5.1)	2.9 (±1.2)	0.8 (±1.6)	4.6 (±1.2)	3.0 (±0.8)	1.1 (±0.9)	2.2 (±1.0)	2.6 (±1.3)	2.8 (±1.1)	0.2 (±0.5)	5.7 (±1.6)	3.3 (±1.7)	2.0 (±1.3)	1.6 (±0.8)	2.0 (±0.8)	0.5 (±0.8)	0.4 (±0.8)	0.0
P. nitidus	**F**	9.3 (±4.3)	15.8 (±1.7)	14.7 (±2.1)	3.2 (±1.2)	2.2 (±1.0)	3.1 (±1.8)	4.0 (±1.4)	0.0	0.0	0.0	0.0	1.4 (±0.6)	7.3 (±3.3)	24.3 (±3.1)	5.2 (±3.6)	1.0 (±2.3)	0.0	0.0	1.7 (±0.6)	5.8 (±2.9)	1.2 (±1.6)
	M	4.2 (±2.1)	23.7 (±3.0)	15.8 (±3.1)	2.9 (±0.5)	2.3 (±0.7)	1.2 (±1.1)	4.2 (±1.5)	0.0	0.1 (±0.2)	0.0	0.0	1.5 (±0.2)	2.3 (±0.5)	26.1 (±2.1)	8.2 (±2.2)	0.0	0.0	0.0	0.0	7.2 (±1.0)	0.3 (±0.6)
S. australis	**F**	11.2 (±3.9)	17.7 (±1.4)	14.6 (±2.1)	5.4 (±1.7)	0.3 (±0.4)	0.0	2.9 (±0.6)	0.0	0.0	0.0	0.0	1.4 (±0.5)	2.2 (±1.3)	7.2 (±1.7)	19.1 (±4.7)	0.0	1.1 (±0.4)	0.7 (±0.6)	1.8 (±0.8)	7.7 (±1.6)	6.7 (±1.4)
	M	12.0 (±3.2)	23.2 (±4.9)	12.8 (±2.1)	3.8 (±1.5)	0.0	0.0	4.0 (±0.8)	0.0	0.4 (±1.0)	0.0	0.0	0.1 (±0.2)	0.8 (±1.1)	5.1 (±1.4)	21.7 (±4.8)	0.0	2.1 (±0.4)	0.5 (±0.6)	0.3 (±0.5)	5.6 (±1.1)	7.7 (±1.3)
S. piliferus	**F**	8.8 (±5.2)	17.1 (±8.5)	23.3 (±6.6)	6.5 (±4.2)	0.0	0.0	0.0	0.0	0.0	0.0	0.9 (±1.4)	0.0	2.5 (±1.3)	8.9 (±3.8)	1.6 (±1.4)	0.0	0.3 (±0.7)	1.1 (±1.3)	0.7 (±1.2)	21.2 (±12.4)	7.1 (±5.3)
	M	8.2 (±5.6)	30.4 (±5.8)	23.4 (±10.5)	2.6 (±3.0)	0.0	0.0	0.0	0.0	0.0	0.0	1.0 (±1.3)	0.0	1.9 (±1.9)	8.0 (±2.3)	0.6 (±1.5)	0.0	0.9 (±1.6)	3.1 (±2.7)	1.5 (±2.1)	15.0 (±2.4)	3.4 (±2.2)

Chapter 7
Thesis conclusions

In central South Island, three endemic species of New Zealand alpine grasshopper coexist on mountain slopes. *Brachaspis nivalis* is more abundant in rock/scree habitats whereas *Sigaus australis* and *Paprides nitidus* are more abundant in the mixed herbfields and tussock, but all three species co-occur in the habitats that contain both rock/scree and vegetation. Under ecological theory of competitive exclusion (Hardin 1960), we might expect these closely related sympatric species to show some evidence of resource-partitioning and species-specific cognitive abilities. This thesis focused on exploring how these three species might coexist by seeking evidence of species-specific differences in their diet and morphology and exploring the mechanisms they use for food and mate recognition.

Tools used in the study of chemical ecology

Many insects rely on chemical cues to locate and recognize their food and mates, and the majority of chemical communication systems in acridid grasshoppers are focused on economically important species, primarily locusts (Chapter 2). This is the first study to explore chemoreception in the New Zealand endemic Catantopinae grasshopper radiation. Chemoreception in insects can be explored using a combination of methods including microscopic observation of sensory organs (i.e., sensilla) using a scanning electron microscope (SEM) and chemical and electrophysiological analyses using a gas-chromatograph coupled with mass-spectrometry (GC-MS) and gas-chromatograph coupled with an electroantennographic device (GC-EAD) and electroantennogram (EAG). Function of sensilla (e.g., taste, olfaction) can be inferred from their morphology, and a high abundance of sensilla is often associated with high sensitivity (Nakano et al. 2022). GC-EAD and EAG record the types and strength of olfactory responses by exposing insects or their sensory organs (e.g., antennae) to the chemicals found in their host plants or mates.

Therefore, the combined use of these tools allows us to test what cues (taste or olfaction) insects are reliant on, chemicals they can perceive, and whether sensilla abundance is linked to olfactory sensitivity.

In this thesis, some differences among species and between sexes were observed, especially differentiating *B. nivalis* from *S. australis* and *P. nitidus* in terms of sensilla abundance (Chapter 4), olfactory sensitivity (Chapter 5) and CHC composition (Chapter 6) which may result from different selection pressures in their habitat (i.e., rock/scree vs. vegetated habitat). Moreover, sexual and species differences in CHC profiles suggest the possibility of a species-specific mate recognition system which may prevent hybridisation and thus facilitate co-occurrence. It is still unknown whether grasshoppers perceive CHCs through olfaction or contact-chemoreception, and further studies involving behavioural, auditory and visual analyses are required to fully understand the sexual communication systems in New Zealand grasshoppers.

Resource partitioning, grazing pressure and native grasslands

Ecological theory suggests closely related species are able to coexist if they partition their resources including habitat and diet (Hardin 1960). Despite this, the three species of New Zealand alpine grasshoppers showed similarity in their diet and olfactory responses to their food plant smell (Chapter 3 & 5) which was unexpected. Co-occurrence of multiple species seems common in many grasshopper assemblages with a high niche overlap (Joern 1979a; Joern and Lawlor 1981; Behmer and Joern 2008; Ibanez et al. 2013b) which may be a product of complex interactions between climatic variables, plant availability, herbivores, predators, and consumer-resource cycles. Modelling of co-occurring competitors suggests that variation or cycles of resources can result in sympatry of herbivores with a high resource

overlap, particularly when the competing species eat at different rates (Abrams and Holt 2002). It is also possible that resource partitioning may be occurring at macronutrient level rather than which plant groups are eaten or not eaten as observed in North American and European grasshoppers (Behmer and Joern 2008; Ibanez et al. 2013b).

Lack of diet specialisation in New Zealand alpine grasshoppers could also be associated with their alpine habitats, as generalists seem more common than specialists in alpine areas (Haslett and Salzburg 1977; Ohler et al. 2020; Pitteloud et al. 2021). For example, in the European Alps an increase in elevation was associated with a decrease in diet specialisation among orthopteran species (Pitteloud et al. 2021). This was explained by high environmental stochasticity (temperature, humidity, irradiation) in alpine habitats which can cause seasonal and yearly fluctuations of available plants and thus specialising could risk their existence. Moreover, lower defences in alpine plants associated with poor soil nutrients make it unnecessary for alpine insects to be specialised on particular plants (Haslett and Salzburg 1977; Ohler et al. 2020; Pitteloud et al. 2021). However, low chemical defence is often associated with higher levels of physical defences (Fernandez-Conradi et al. 2022) requiring alpine grasshoppers to have strong mandibles and bite.

The study of New Zealand alpine grasshoppers' gut contents is more than 50 years old (Watson 1970) and thus this thesis provided a good opportunity to re-analyse their diet and observe whether there are any changes in diet composition. Past studies suggested that New Zealand alpine grasshoppers might impose appreciable grazing pressure on endemic grasslands at high elevations (White 1974a, 1975a, b, 1978), which could eventually lead to loss of alpine diversity. Moreover, 10–18 % of the diet of these alpine grasshoppers comprised material interpreted as flowers (Watson 1970); by eating the reproductive structures of particular plant species grasshoppers might have a significant impact. As

inferred from mandible analysis, microhistological and genetic analyses of gut contents (Chapter 3) and feeding trials (Chapter 5), males and females of all species preferred to eat dicot herbs and shrubs over hard tussocks. Although grasshoppers showed some preference for particular plant species, most of the plant species that they feed on are not at risk of extinction or declining (de Lange et al. 2013; 2023). The invasive exotic weed *Hieracium*, a plant genus that has increased its distribution in the past 50 years in the New Zealand high country (Meffin 2010; Steer and Norton 2013; Jensen et al. 2019) was also detected in grasshoppers' gut contents (Chapter 3). If the grasshoppers are selectively feeding on *Hieracium* they may contribute to preservation of native plant communities in New Zealand alpine area.

Future challenges

Global warming is expected to cause changes in distribution, abundance and biotic interaction in plants and animals (Giron et al. 2018), especially for cold-adapted alpine specialists. This was supported by studies based on modelling (Koot et al. 2022; Meza-Joya et al. 2023), long-term observations of abundance and distribution of alpine species (Illich and Zuna-Kratky 2022), and experimental warming studies (Birkemoe et al. 2016). For example, increased abundance and distribution of some widespread generalist orthopteran species but restricted dispersal in alpine specialists were observed in the Central Alps between 1991 and 2020, which may have been the result of a 1.6°C increase in the average summer temperature in 30 years (Illich and Zuna-Kratky 2022). Increase in competition could also occur as a result of global warming, due to the shifts of plants and animals' distributions to higher elevations. My research detected no sign of diet segregation in the sympatric New Zealand alpine grasshopper species, which may suggest that competition for

food is not intense. However, as climate change progresses their habitat is expected to be restricted (Koot et al. 2022) and therefore increased risk of competition could eventually lead to population extinctions.

The ability of species to persist depends on availability of suitable habitat, dispersal capacity and genetic and phenotypic diversity. High intraspecific diversity was observed in *B. nivalis*, *S. australis* and *P. nitidus* (Meza-Joya et al. 2023) and lack of specialization to particular plant species (Chapter 3 & 5) may allow them to survive in changing environments and sophisticated communication mechanisms (Chapter 6) could maintain species diversity by avoiding hybridisation. However, these species are all flightless and past studies showed the average dispersal distance recorded during 1 year as an adult is 70–150 m (White 1974b). Low dispersiveness and presence of environmental barriers (e.g., forests, valleys) will mean that colonising new habitat may require human transportation suggesting low resilience to climate change. Further understanding of their dispersal ability, habitat, feeding ecology, and sexual communication systems will help us understand their resilience to changing environments.

Future directions

The work presented in this thesis gave insights into food plants, chemoreception, and chemical profiles in three New Zealand alpine grasshoppers, but has also raised further important questions relating to the way they and their habitat has evolved:

- To what extent are size, toughness, taste and/or appearance involved in the food choices of the grasshoppers?
- Are sexual and species differences in mandible structures associated with adaptation to the size and toughness of particular plant structures or species?

- If grasshoppers are excluded from alpine habitats do herb species increase and outcompete grass species?
- Do plant-derived chemicals act as grasshopper attractants or repellents?
- Do grasshoppers perceive CHC cues through olfaction or contact-chemoreception and are other cues (e.g., vision, acoustic) involved in mate selection?

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