Invasive paper wasp (*Polistes chinensis*) ecology and invertebrate conservation on Onetahua (Farewell Spit)

by

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General abstract

Social insects are among the most ecologically and economically damaging, and abundant invasive species worldwide. Invasive paper wasps in Aotearoa have been shown to cause significant declines in native insect populations though predation, with cascading ecological impacts. No effective control methods for paper wasps are currently available. One such wasp, *Polistes chinensis*, can become especially abundant in coastal scrub and low-elevation grassland habitats. Together these habitats are estimated to house 60% of Aotearoa's threatened native Lepidopterans alone.

The Onetahua (Farewell Spit) Nature Reserve is a highly protected coastal site encompassing nationally rare habitat types and several at-risk plant and invertebrate species. During a 2019 BioBlitz, paper wasp abundance at this site was noted as likely threatening these diverse invertebrate communities. To inform decision-making on the potential need for local management of these wasps, I aimed to assess their distribution and population dynamics and identify species in their prey community. Over two austral summers, 2020 – 2021 and 2021 – 2022, I surveyed paper wasp colonies on Onetahua and tracked colony survival and development rates. Using wasp larvae gut samples, I conducted a CO1 metabarcoding analysis to identify prey species over a temporal gradient. During data and sample collection, I observed two species of entomopathogenic fungi fatally infecting *P. chinensis* wasps at their nests. Taking samples of these fungi, I identified each species using morphological and molecular methods, attempted to quantify their prevalence in the studied wasp population, and conducted infection bioassays with caged wasp nests to test fungal pathogenicity.

From colony surveying and monitoring, I found that approximately 20% of all spring colonies survived through each summer. Colony development rates indicated that wasp predation pressure likely peaked in February of each year, and I estimated that average colony density in February 2022 was approximately 23 colonies ha^{-1}. The most ubiquitous prey species identified were several endemic cicadas and numerous Lepidopteran species, including common copper butterflies (*Lycaena* spp.). However, ~60% of unique taxa could not be identified using this barcoding method, likely due to the absence of barcodes for these species in reference libraries. I conclude from this result that metabarcoding analysis is probably not suitable for the identification of many species of conservation significance, as these taxa are likely to be rare, understudied, or undescribed species. A more comprehensive unification of

taxonomy and genetic sequencing of invertebrates would be beneficial to improve the useability of such analyses.

Fungal pathogens were estimated to have infected wasps in ~3% of all surveyed colonies. I identified the fungal species observed as *Beauveria malawiensis* and *Ophiocordyceps humbertii*. In bioassays I successfully infected healthy *P. chinensis* colonies with *B. malawiensis*, with significantly increased wasp mortality rates and conidiogenesis in treatment nests. These fungal species may have potential for development as biological control agents for *Polistes* wasps in Aotearoa and elsewhere around the world.

I did not find sufficient evidence to warrant diversion of current conservation resources to high-intensity manual control of *P. chinensis* on Onetahua. However, I do recommend that repeated monitoring of paper wasp and prey species' relative abundances be carried out at Onetahua and similar sites. Determination of population trends in these species could be invaluable for future management of *Polistes* wasps, if and when efficient control methods are eventually developed.

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Statement of authorship

I hereby declare that this thesis is my own work and that all sources paraphrased or referred to have been properly acknowledged in the references.

Chapter 2 author contributions: A.R., P.J.L., and R.T. developed research aims and study design; A.R. conducted data and sample collection in the field with assistance from E.B-P. and S.P., and input from R.A.M.; A.R. conducted sample preparation with advice from M.W.F.H.; M.B. conducted molecular laboratory work; A.R and A.F. conducted data analysis; J.H. provided statistical advice; A.R. wrote the draft manuscript; P.J.L. reviewed and assisted with editing of the manuscript.

Chapter 3 was published in *Insects* on 12 October 2022. Author contributions: A.R. and P.J.L developed research aims and study design; A.R. conducted microscopy work, managed wasp husbandry, and carried out assays; M.B. conducted molecular laboratory work; A.R. and M.B. conducted data analysis; A.R. wrote the draft manuscript; P.J.L. and M.B. assisted with review and editing of the manuscript.

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Chapter 1: General introduction

1.1 Social insects as invasive species

Invasive species are one of the primary drivers of biodiversity loss around the world, through direct and indirect effects on recipient ecosystems (IPBES, 2019). Increasing levels of globalisation, ongoing climate change, and other anthropogenic pressures can work in synthesis to both increase the occurrence of new species invasions and exacerbate their ecosystem impacts (Pyšek et al., 2020). Island ecosystems, with high levels of endemism and flora and fauna whose recent evolutionary history has been in isolation, are known to be particularly vulnerable to the effects of invasive species (Bellard et al., 2017; D'Antonio & Dudley, 1995; Kier et al., 2009; Reaser et al., 2007). Aotearoa exemplifies such an ecosystem, having a relatively low species richness for many groups of organisms compared to continental nations. For example, native social Hymenopterans in New Zealand are represented only by the eleven endemic ant species (Ward, 2005). The resulting lack of competition for introduced social Hymenopterans may be a contributing factor for invasive wasps in Aotearoa having once reached global record densities (Thomas et al., 1990).

Many of the most significant and damaging invasive species worldwide are among the eusocial insects, despite the very small proportion of insects with eusocial life histories (Beggs et al., 2011; Bertelsmeier, 2021; Kenis et al., 2009). One probable explanation for this occurrence is that a eusocial lifestyle is frequently associated with phenotypic plasticity, conferring advantages in dispersal to, survival and reproduction in, and adaptation for new environments (Manfredini et al., 2019).

Five species of social Vespid wasps are recognised as invasive species in Aotearoa. Alongside the well-known common and German wasps (*Vespula* species), there are three species of paper wasps present. These are the Australian, Asian, and European paper wasps *Polistes humilis*, *P. chinensis*, and *P. dominula*, respectively (Beggs et al. 2011, MPI 2016). *Polistes humilis* was the first arrival in the 1880s, and today is established in northern and some eastern areas of the North Island (Clapperton et al. 1989). The Asian paper wasp (*Polistes chinensis*), native to north-east Asia, was first recorded in the Auckland region in 1979, and was likely introduced accidentally in two separate events (Tsuchida et al., 2014). This species has since gained an almost country-wide distribution (Clapperton et al., 1989; Clapperton et al., 1996), with its dispersal likely to have been facilitated by human movement (Schmack et al.,

2020). The most recent paper wasp invasion was of *P. dominula*, which was officially recorded in 2016 but possibly present as early as 2011 (MPI 2016, McGruddy 2021). Due to their rapid spread and the high productivity of this species, *P. dominula* may be outcompeting other paper wasps in Aotearoa in some environments, potentially presenting a more significant ecological threat (Gamboa et al. 2004, Roets et al. 2019, McGruddy 2021). However, survey work to date appears to indicate that *P. dominula* has a preference for urban habitat, and in remote coastal areas *P. chinensis* is likely to remain more abundant (McGruddy 2021).

1.2 Paper wasp biology and ecological impacts in Aotearoa

Polistes chinensis' typical native habitat is lowland and coastal areas of herbaceous or shrubby vegetation (Suzuki 1978). They are rarely found at high elevations or under forest canopy cover, likely due to climatic tolerances (Clapperton & Dymock, 1997; Clapperton et al., 1996). Their paper nest combs are constructed from woody plant fibres and a proteinaceous oral secretion is used for adhesion and waterproofing (Kudô, 2000). These delicate combs are small and inconspicuous, usually close to the ground amongst dry, twiggy vegetation, to which they are attached by a short stem called a petiole or pedicel. As *P. chinensis* is not territorial, nests may occur in very high densities, and foraging areas of different nests frequently overlap (Clapperton & Lo, 2000; Clapperton et al., 1996; Miyano, 1980). Nest densities typically range between 20 and 40 nests ha^{-1} in favourable habitat, although densities as high 210 nests ha^{-1} have been recorded in their invaded range (Clapperton, 1999; Ward & Morgan, 2014).

Colony development in *Polistes chinensis* can be distinguished in three distinct stages: the 'founding' phase; 'superindividual' or 'worker' phase; and 'social' or 'reproductive' phase (Kudô, 2000; Miyano, 1980; Sumner & Cini, 2021; Yoshikawa, 1962). The founding phase begins with nest initiation by a single fertilised female in spring, as early as late September or early October in Aotearoa (Clapperton and Dymock 1997). Until the emergence of the first foundress-reared adult, the foundress is the sole adult in the colony, and is highly vulnerable to predation, nest parasites, and intercolonial competition (Kudô, 1998). The first adults emerging from their pupal cells begin the superindividual phase. These foundress-reared adults will start leaving the nest to forage and hunt a few days after emergence, and in their native range live for an average of 38 days (Miyano 1980). With colony tasks divided among more adults, both the size of the comb and the nest population can expand exponentially in this phase (Clapperton & Dymock, 1997). Finally, the social phase begins with the emergence of male

reproductive adults, shortly followed by reproductive-destined females called gynes (Suzuki, 1986). After mating occurs in autumn, the nest will finally dissolve, with fertilised gynes preparing to overwinter, and males dying off (Miyano, 1980). Fertilised females will disperse and hibernate through winter in sheltered cavities, sometimes communally, until the next spring when the cycle resumes (Miyano 1980, Clapperton et al. 1996).

Nest failure rates are high, especially in the founding stage. A study from *P. chinensis'* native range found that 57.4% of colonies survive until natural dissolution of the colony in autumn, with 30.2% of all colonies failing during spring, usually due to loss of the spring foundress (Miyano 1980). Colony death at later stages may be caused by predation of the brood, and in the wasps' native range, parasitisation by moths and other specialist parasites (Strassmann 1981). Like all organisms, *Polistes* wasps are also susceptible to microbial pathogens and diseases (Mhlongwe, 2018; Somavilla et al., 2020a).

Adult paper wasps hunt for live invertebrate prey to provide to their larvae, which function as the protein digesters of the colony. As invasive species in New Zealand, *Polistes* wasps have been found to substantially decrease native and introduced butterfly populations, which can produce significant cascading effects on plant productivity (McGruddy et al., 2021b). They may also impact ecosystems via competition for resources or by impacting regular plant pollination (Clapperton, 1999). Recent studies using molecular methods to examine the paper wasp prey community in Aotearoa have identified taxa impacted, which includes Lepidopterans (butterflies and moths), cicadas, hoverflies, craneflies, thrips, mites, and spiders (Ward and Ramón-Laca 2013, Todd et al. 2015, Lefort et al. 2020, Howse et al. 2021). In coastal areas, one study indicated that native taxa were estimated to constitute 62% of the total prey diet (Ward and Ramón-Laca 2013).

Currently there are no methods available for the efficient landscape-scale control of paper wasp populations. Individual *Polistes* nests can be identified and manually treated and destroyed with a general insecticide. However, the small and often well-camouflaged nests, potentially in very high densities, make this method impractical and likely ineffective at a landscape scale. Previous research has shown trapping to be unsuccessful (Toft & Harris, 2004). Carbohydrate-based toxic baits and lures are not considered a viable option due to the attraction of non-target insects, and moreover, paper wasps are not attracted to carrion protein sources (Richter, 2000). Currently, biological control has been the recommended research avenue for paper wasp control, as their nest construction behaviours make them a

much better target than other Vespid wasps for biological controls such as parasitoids, predators, and pathogens (Brown, 2021). Biological control agents should be species-specific, and if such an agents can be found for *Polistes*, may be a more efficient method of control than manual removal (Lester et al., 2013).

1.3 Case study site: Onetahua (Farewell Spit)

I used Onetahua (Farewell Spit) as a case study site at which to collect data on invasive *P. chinensis* ecology. Onetahua is a 25 km-long barrier sand spit reaching east from its base near the Cape Farewell headland, the northernmost point of New Zealand's South Island. The climate of this site is heavily influenced by its marine surroundings, with high humidity and strong westerly winds, and the landscape consists of dune wetland and low-growing saline vegetation. As a result of previous land uses, much of the native vegetation has been destroyed and degraded (Kelly, 1991; Petyt, 1999). Subsequent mass planting and invasion of non-native species such as marram grass (*Ammophila arenaria*), tree lupin (*Lupinus arboreus*), gorse (*Ulex europaeus*), and blackberry (*Rubus fruiticosus*) have slowed the re-colonisation of native vegetation in many areas (Bell et al., 1961; Brown, 1978).

The spit and associated mudflats constitute a Department of Conservation Nature Reserve (Reserves Act Government, 1977; Conservation Act Government, 1987), designated Ramsar wetland (Ramsar, 1971), and international Flyway site under the East Asian-Australasian Flyway Partnership (EAAFP, 2018), and are the yearly feeding and breeding grounds for many migratory bird species. Onetahua has been recognised for many years as an important ecological site for bird life (Bell et al., 1961; Robertson & Dennison, 1979), and more recently as a hotspot of biological diversity. Several rare species have been found to occur locally, especially among endemic coastal plants and invertebrates. For example: the sand spike sedge (*Eleocharis neozelandica*); sand spurge (*Euphorbia glauca*); sand daphne (*Pimelea arenaria*); pīngao (*Desmoschoenus spiralis*); *Austrocidaria arenosa* (Geometrid moth); an undescribed cave wētā (*Isoplectron* sp.); an undescribed Therevid fly (*Megathereva* sp.); an undescribed *Notoreas* sp. moth; and an undescribed copper butterfly (*Lycaena* sp.) (Deans, 1992; Kelly, 1991; Patrick & Patrick, 2019; Toft, 2020; Trewick & Morgan-Richards, 2020).

The presence of *Polistes chinensis* on the spit was recently highlighted as of conservation concern (Toft, 2020), prompting local conservation organisations to investigate the ecological impact of paper wasps in the area. Onetahua encompasses high-priority habitat

in which to investigate the ecological impacts of *P. chinensis* and establish baselines of colony and wasp abundance (Ward & Morgan, 2014). Any potential for population control of this species on Onetahua could also be desirable for human health and safety, as it is a popular tourist site in the summer months. While not typically aggressive, paper wasps will defend their nest if sufficiently disturbed, can inflict a painful sting, and in rare cases more serious health issues such as allergic reactions and anaphylaxis.

1.4 Aims and overview

The overall goal of this project was to gather ecological data that will aid in understanding of paper wasp population dynamics and determine if they are a threat to invertebrate conservation on Onetahua; to establish a current baseline of *Polistes* abundance for ongoing population monitoring; and to and inform future control measures if deemed necessary.

Using the Onetahua/Farewell Spit Nature Reserve as a study site, I collected data on *Polistes chinensis* colony ecology, made observations of their foraging behaviours in the habitat, and collected samples of wasp larvae. In chapter two, I used this data to estimate wasp colony density and estimate the period of the nesting season with the highest level of wasp predation pressure. Using the collected larvae samples, I conducted a DNA metabarcoding analysis of the larvae gut contents to identify species in the wasps' prey community. By considering both the colony development and diet data, in this chapter I have presented a list of known species likely to be facing the highest degree of predation pressure from *P. chinensis*, and discussed potential implications for invertebrate conservation.

During data and sample collection, I observed several wasp nests with adult wasps that had become infected with unknown entomopathogenic fungi species. In chapter three I identified the two species of fungi found in infected specimens, using morphological and molecular methods. I also attempted to infect live, healthy paper wasp colonies with each fungus in bioassays, to confirm the pathogenicity of these species to *Polistes chinensis*.

In the final chapter I have synthesised the findings of previous chapters and discussed their relevance within the current body of knowledge on invasive paper wasps in Aotearoa. Finally, I have assessed my findings in respect to the case study site of Onetahua, what they mean for invertebrate conservation within the Nature Reserve and other areas of similar habitat, and suggested possible suitable courses of action for future work.

Chapter 2: Population dynamics and prey community of the invasive paper wasp *Polistes chinensis* in a protected coastal habitat

2.1 Abstract

An invasive species and voracious predator of Aotearoa's native insect fauna, the Asian paper wasp (*Polistes chinensis*) is an important study species in the interests of invertebrate conservation. I examined paper wasp ecology, colony productivity, and survival rates in Onetahua (Farewell Spit) Nature Reserve and analysed the composition of the wasps' prey community. Over two summer seasons, I surveyed paper wasp colonies and monitored colony development weekly. I used samples of wasp larvae gut contents collected over a temporal gradient to conduct a CO1 metabarcoding analysis. I found that only ~20% of the monitored colonies each year survived until late summer, with high rates of colony mortality in late spring and early summer. Average colony development rates indicated that the colony phase in which the greatest predation pressure is exerted on the prey community likely occurs in February. Prey species identified most frequently from samples collected in February were endemic cicadas and several Lepidopterous species, including *Lycaena* spp. No species of explicit conservation concern were identified from this dietary analysis. However, 60% of unique taxon sequences retrieved from samples could not be identified to genus or species, likely due to the absence of reference barcodes for rare species. These sequences may represent a group of understudied species, potentially highly endemic or localised to the study site. Long-term monitoring of the relative abundances of *Polistes chinensis* and their prey species on Onetahua and similar areas is recommended for determining the ecological impacts of *P. chinensis* predation.

2.2 Introduction

Given the small proportion of all insects that are social, they are substantially overrepresented among invasive insect species (Lowe et al., 2000; Manfredini et al., 2019). Insects' small size makes them prone to human-assisted dispersal, and the eusocial lifestyle allows colonisation of new areas sometimes by a single fertilised individual (Moller, 1996). The phenotypic plasticity associated with sociality facilitates quick adaptation to new environments (Manfredini et al., 2019; Richards et al., 2006). Coupled with the omnivorous and often predatory diet of many

social insects, these and more factors can result in catastrophic and cascading impacts on recipient ecosystems and agricultural economies (Beggs & Jo, 1999; Beggs & Wardle, 2006; Brockerhoff et al., 2010; Holway et al., 2002; McGruddy et al., 2021b).

Onetahua (Farewell Spit) Nature Reserve, Aotearoa, is a barrier sand spit with highly valued geomorphology, ecology, and history (Petyt, 1999; Tribe & Kennedy, 2010). The site has been recently recognised for bearing an especially rich and diverse invertebrate community (Patrick & Patrick, 2019; Toft, 2020; Trewick & Morgan-Richards, 2020), likely in part due to its unique landscape incorporating rare habitat types and plant species (Deans, 1992; Kelly, 1991). Native butterflies reported from Onetahua include red and yellow Admirals (*Vanessa gonerilla* and *V. itea*), copper butterflies (*Lycaena spp.* and *L. rauparaha* species complexes), and common blue butterflies (*Zizina otis labradus*) (Petyt, 1999). The site also comprises a diverse community of moths, including two species considered threatened: *Austrocidaria arenosa* (Geometridae; 'Declining') with host plant *Coprosma acerosa* (also 'Declining'); and an undescribed *Notoreas* species (Geometridae; 'Nationally vulnerable') with host plants of *Pimelea* sp. – most likely *P. villosa* on Onetahua, also 'Declining' – (de Lange et al., 2017; Deans, 1992; Hoare et al., 2017; Patrick & Patrick, 2019). As such, Onetahua is a high-priority site for research into invertebrate conservation.

One invasive insect that has colonised the reserve is the Asian paper wasp, *Polistes chinensis*. This wasp is an Arthropod predator, considered somewhat a Lepidoptera (butterfly and moth) specialist, though it will hunt a wide range of prey (Lefort et al., 2020). Though its colony sizes are relatively small, *P. chinensis* nests can occur in some locations at very high densities (Clapperton, 1999; Clapperton et al., 1996; Ward & Morgan, 2014). Like related species in Aotearoa, *P. chinensis* is likely causing significant impacts on local invertebrate communities, especially populations of native Lepidoptera (Beggs & Jo, 1999; Clapperton, 1999; Howse et al., 2022; Lefort et al., 2020; McGruddy et al., 2021b; Ward & Ramón-Laca, 2013), with native taxa constituting 62% of paper wasp diets in coastal areas (Ward & Ramón-Laca, 2013).

A single successful *Polistes chinensis* nest may produce many new potential foundresses. However, only a relatively small proportion of young nests survive through the vulnerable founding stage and go on to produce fertilised females. In Auckland and Northland, previous research has found the average colony survival rate to be 22 – 25% by late summer (Clapperton & Dymock, 1997), a rate considerably lower than in the wasps' native range in Japan, where

colony survival can be as high as 41% (Miyano, 1980). This discrepancy in colony survival may be due to many factors, such as the genetic bottleneck in the arrival of *P. chinensis* to New Zealand (Tsuchida et al., 2014), and differences in habitat and climate. Temperature and rainfall are both known to have significant effects on wasp colony development and productivity (Jeanne & Morgan, 1992; Kudô, 2000), which may impact overall competitive ability and survival. As Aotearoa spans a range of latitudes, there is a substantial latitudinal gradient in climate between different regions (Mackintosh, 2001) as well as a range of habitat types, and so colony dynamics may differ slightly from region to region.

The three main phases of colony development in *Polistes chinensis* are associated with differing proportions and overall numbers of individuals at each life stage, and thus different resource requirements. In the founding phase, with only a few vulnerable brood, the foundress hunts only sparingly (Suzuki, 1978), and will preferentially feed the strongest larvae in order to raise these to maturity as fast as possible (Hoshikawa, 1981). She will often cannibalise eggs and other larvae to this end (Kasuya et al., 1980; Kudô & Shirai, 2012; Miyano, 1980, 1998). After adults emerge, beginning the superindividual phase, the nest grows exponentially and the numbers of hungry larvae, as well as the number of adults available to hunt for them, increases (Suzuki, 1978). Finally, the social phase begins when males and reproductive females emerge. At this time, the feeding of larvae becomes de-prioritised, and the majority of extranidal tasks conducted by the colony transitions from protein hunting to foraging carbohydrate foods for the adult wasps directly (Hoshikawa, 1981). Thus, paper wasp colonies might be expected to exert the greatest degree of predation pressure on invertebrate communities during the late superindividual and early social phases. The composition of species in their prey community in a given area is expected to change throughout the nesting season due to the phenology of each individual prey species, as well as variable environmental conditions (Lefort et al., 2020).

The aims of this chapter were: 1) to survey the nesting ecology, including the average colony development and survival rates, of *Polistes chinensis* paper wasps on Onetahua/Farewell Spit; 2) to identify invertebrate species comprising the prey community of these wasps; and 3) to use wasp abundance estimates to evaluate potential threat of predation pressure to the prey species. By comparing the colony dynamics of paper wasps in different regions and habitats, we may improve our understanding of the population dynamics of this invasive species, and the level of biodiversity threat they pose in critical areas such as Onetahua. By identifying species in the prey community, we may learn if *P. chinensis* predation is likely to be posing an immediate

threat to the population of vulnerable native species at this study site. Additionally, in this chapter I will present observations of foraging and hunting wasps that were made while collecting data and samples in the field. The wasps' use of microhabitat or repeated associations with certain plant species or functional groups may help point toward certain species or communities bearing the brunt of predation pressure from these wasps. This work will aid decision makers to assess the necessity for future monitoring and conservation work on Onetahua and similar habitats, and provide a focus for these efforts.

2.3 Materials and Methods

2.3.1 Colony and population dynamics

Data and sample collection for this study was carried out over two austral summers, from late November to late February of 2020 – 2021 and 2021 – 2022. Colony development and survival was measured through weekly monitoring and observation of paper wasp nests. In the first two weeks of study in each year, an approximately 120 ha area within 2 km from the western base of Onetahua (Fig. 2.1) was manually searched for wasp nests, including empty combs, in order to establish a population sample for monitoring. Manual searching was conducted area-by-area (delimited by natural or artificial landforms or markers) by two people walking five to ten meters apart, covering all area. All accessible vegetation and anthropogenic structures that could feasibly house or conceal wasp nests (such as buildings, fences, tall grasses, rushes, shrubs, and trees) were thoroughly searched. The starting sample numbers were 77 nests for the 2020 – 2021 summer, and 251 nests for 2021 – 2022. Upon discovery of a nest, I counted and recorded the number of cells in the comb, whether eggs were present, numbers of larvae and capped cells (containing pupae or larvae preparing to pupate), vacated cells, and number of adult wasps present. I also recorded the GPS location, height from the ground to the nest petiole in centimetres, and plant species to which the nest was attached. In each following week, all nests in the sample cohort that were alive in the previous week were visited and the comb size and colony metrics re-counted. Notes were also taken on the general condition and health of each nest, including the death of one or more wasps from fungal infection, where these events were apparent.

Figure 2.1 The locations of Onetahua and Nelson in Aotearoa, and a site map of Onetahua. The hashed area at the western base of the spit was the study area for weekly colony monitoring, approximately 120 ha in total, consisting of mixed habitat types. The blue dots labelled P1 – P5 are the locations of each 0.5 ha sampling plot. The dotted area indicates approximate areas searched for nests in random walking surveys. Aerial map imagery sourced from LINZ data service [accessed from https://data.linz.govt.nz/].

I combined data from both years of nest monitoring and carried out statistical analysis on colony dynamics and survival in R 4.1.1 (R Core Team, 2020). A Kaplan-Meier survival analysis with a log-rank significance test was used to compare colony survival rates between years, with R packages *survival* and *survminer* (Kassambara et al., 2021; Therneau, 2021). I used Wilcoxon rank-sum tests to compare mean per-nest counts of cells, larvae, pupae, and total adult individuals observed between years in the final monitoring week. Non-parametric tests were necessary in this case due to the data being non-normally distributed.

In a preliminary assessment of wasp distribution and nesting patterns on Onetahua, active visual searching surveys were carried out at nine sites arrayed along the full length of the spit in the 2020 – 2021 summer (Fig. 2.1) (Montgomery et al., 2021). The sites visited were primarily the locations of recognisable, named landscape features, to facilitate communication with the Farewell Spit EcoTours staff who facilitated our transport to locations on the eastern half of the spit. Surveying was carried out by two people walking slowly, approximately 5 to 10 m apart through the vegetation. Search paths were largely dictated by vegetation type and accessibility. For example, open sand pans and dune hollows dominated by *Samolus repens, Selliera radicans,* or *Salicornia quinqueflora* were not searched due to the lack of suitable substrates for *Polistes* nesting, along with permanently wet areas and (rare) canopied areas (Schmack et al., 2020). Otherwise, all vegetation was thoroughly searched within a contiguous area, the size of which was determined by time constraints. GPS coordinates were recorded for all discovered nests, including empty combs, along with notes on the substrate vegetation and nest microhabitat.

In the 2021 – 2022 summer, five 0.5 ha sample plots were randomly placed within the general areas surveyed in 2020 – 2021, using a random point placement tool in QGIS 3.25 (QGIS.org, 2021), and surveyed to estimate average nest density (Fig. 2.1). Coordinates for these plots were located in the field and the perimeters temporarily marked with stakes. Each plot was thoroughly searched by two people for all wasp nests, including empty combs. GPS location, nest and habitat data were all recorded for each nest discovered. Due to logistical limitations, the sample plots could not all be surveyed at the same time, nor repeatedly to determine colony survival. Thus, to make an estimate of the average number of colonies surviving per hectare in late February, I used the number of live colonies found in each sample plot at the time of surveying (n_{Alive}), with the approximate survival probability of colonies at that time point extracted from the Kaplan-Meier analysis for the 2021-2022 summer (P_{Date}), and the overall final survival probability of monitored nests in late February 2022 (P_{End}; Eq. 2.1) (Archer, 1985).

Eq. 2.1 a

$$\boldsymbol{N_{Alive}} = \boldsymbol{n_{Alive}} \times \mathbf{2}$$

Eq. 2.1 b

$$\boldsymbol{N_{Start}} = \frac{\boldsymbol{N_{Alive}}}{\boldsymbol{P_{Date}}}$$

Eq. 2.1 c

$$\boldsymbol{N_{End}} = \boldsymbol{N_{Start}} \times \boldsymbol{P_{End}}$$

Where: P_{Date} = Kaplan-Meier survival probability at survey date (95% CI);

$P_{End} = 0.22\ (0.18, 0.28)$

Eq. 2.1 d

$$\boldsymbol{N_{End}} = \frac{\boldsymbol{n_{Alive}} \times \mathbf{2}}{\boldsymbol{P_{Date}}} \times \boldsymbol{P_{End}}$$

2.3.2 Field observations

To improve our understanding of Asian paper wasp resource use, hunting, and foraging habits, observations of wasp behaviours were made and recorded opportunistically throughout the periods of sample and data collection. Whenever practical, any adult wasps found conducting off-nest tasks were observed, and their behaviours and interactions with flora and fauna recorded. On each occasion when found, a single wasp was followed and continually observed for as long as possible, usually until the individual was lost. Observations were also made of wasps undertaking on-nest tasks, particularly wasps returning to the nest with flesh boluses from prey items, and activities relating to structural nest maintenance and repair.

2.3.3 Diet analysis

Sample collection

Attaining samples that will reliably represent the diversity of prey consumed by paper wasps must involve sampling the diet of the wasp larvae specifically, as the larvae are the protein digesters of a wasp colony. Adults provide larvae with flesh food and consume the protein-rich saliva produced by the larvae in return to meet their dietary protein needs (Kudô & Shirai, 2012; Kumano & Kasuya, 2001). To examine the protein diet of the Onetahua paper wasp population I took live, whole, healthy larvae from active paper wasp colonies. These nests were randomly selected from a pool of previously discovered nests within the study area using random number generation. If a selected nest did not have larvae of a suitable size for

sampling, the next closest nest in physical distance was sampled instead. Larva samples were collected in mid-December, -January, and -February of the 2020 – 2021 austral summer to detect intra-seasonal variation in prey quantity and diet composition. For each nest sampled in each month, two fourth or fifth instar larvae were extracted from their nest using forceps and placed in 1.5 mL tubes with 90% ethanol. The forceps were cleaned and sterilised with ethanol between nest samples. Sixteen to 17 nests were sampled each month for a total of 98 larvae from 37 unique nests (Table A.1). Samples were immediately stored at -18 °C before being transported to the laboratory and stored at -80 °C until further processing. The frozen larvae were dissected, and the gut contents isolated.

DNA extraction, PCR, sequencing, and identification

I extracted DNA from individual larva guts, following the protocol described in Howse et al. (2022). Each sample was homogenised in a Precellys Evolution homogeniser (Bertin Technologies, France) with 1 mL GENEzol DNA Plant Reagent (Geneaid, Taiwan), 5 µL β-mercaptoethanol, and two stainless steel beads per tube. The DNA was isolated with chloroform isoamyl alcohol, precipitated using isopropanol, purified with a 70% ethanol wash, and resuspended in 100 µL nuclease-free H_2O. I measured DNA concentrations using a NanoPhotometer NP80 (Implen, Germany). Sample PCR amplification and DNA sequencing was provided by Custom Science (Auckland, New Zealand). I used PCR primers targeting the mitochondrial gene cytochrome oxidase 1 (CO1) developed by Zeale et al. (2011) (ZBJ-ArtF1c: GATATTGGAACWTTATATTTTATTTTTGG and ZBJ-ArtR2c: WACTAATCAATTWCCAAATCCTCC). These primers are specific to Arthropoda and amplify a 157 base-pair (bp) region of the mitochondrial gene. They have been successfully used to identify Arthropod prey in bat faecal samples (Alberdi et al., 2020; Zeale et al., 2011) and *Polistes* larval gut samples (Howse et al., 2022). Seventy two of the 98 larval gut samples from which DNA was extracted amplified successfully. Next, DNA samples were sequenced and generated 150 bp paired-end reads on a HiSeq X platform (Illumina, USA).

Taxonomic assignment was performed using Basic Local Alignment Search Tool (BLAST) 2.11.0, using BLASTn searches against the National Center for Biotechnology Information (NCBI) nucleotide database (nt), retrieved on 27 April 2022 (Camacho et al., 2009; Sayers et al., 2021). First, the *rmdp* command in *SeqKit* 0.13.2 (Shen et al., 2016) was used to remove duplicated sequences in each FASTA sample file, while retaining the original number of sequences. I ran

BLASTn using the deduplicated files as input, selecting the best hit based on the highest bit score. The output of BLASTn was specified to include taxonomic identification number from the Taxonomy Database (taxid) retrieved on 27 April 2022 (Sayers et al., 2021). Full taxonomic classification (i.e. order, family, genus, species) for each hit was retrieved from the taxid in BLAST outputs using *Edirect* 13.9 (Kans, 2022). I reanalysed sample data from Howse et al. (2022) using this methodology, which was necessary because the reference databases have been updated since their work. Raw data from both studies can be retrieved from the NCBI SRA database under Bioprojects PRJNA856715 and PRJNA856051.

The BLAST output and taxonomic data was sorted and cleaned to improve confidence in taxon identification and reduce the impact of false positives, following protocols similar to those outlined in Howse et al. (2022). Alignments less than 100 bp in length, sequences with BLAST matches less than 98% similarity, and matches with less than 10 sequences retrieved per sample were removed (Vesterinen et al., 2018). All reads assigned to *Polistes* spp. were also removed, as these likely remain from the wasp larval tissue, or from oophagy or larval cannibalism via adult wasps, which is a known phenomenon for these wasps (see Fig. 2.6) (Kudô, 1998; Kudô & Shirai, 2012; Miyano, 1980, 1998). It would have been impossible to differentiate host tissue reads from cannibalism in our analysis. The remaining reads were organised into a taxonomic list of the wasp diet community, and each taxon checked against the New Zealand Organism Registry (NZOR, 2012c), Global Biodiversity Information Facility database (GBIF.org, 2021), and iNaturalistNZ website (https://inaturalist.nz/) for recorded presence in New Zealand, at the genus or family level of each taxon. For assigned taxa recorded as present in New Zealand, species or genus biostatus was also determined. Organism "biostatus" is a standardised system of terms indicating the occurrence and origin of a species or higher classification in New Zealand, for example 'endemic,' 'native,' or 'exotic' (Martin, 2007).

Data analysis

To assess the diversity of the wasp diet sampled from each site, species accumulation curves were constructed using the 'specaccum' function from the *vegan* package (Oksanen et al., 2022) in R 4.1.1 (Team, 2020). I used the rarefaction method to assess the completeness of the diet community from the samples from each site. Venn diagrams were plotted for the

Onetahua diet community from each sampling month across the season, to visually assess intra-seasonal prey diversity.

2.4 Results

2.4.1 Colony and population dynamics

A total of 493 and 522 paper wasp nests were discovered and recorded across all sites on Onetahua in the 2020 – 2021 summer and the 2021 – 2022 summer, respectively. Nests were found to be present in almost all sites visited within areas of suitable nesting vegetation and moisture levels. The vegetation most utilised for nests was low-growing shrubs (<1 m), such as *Muehlenbeckia complexa* (pohuehue), *Coprosma acerosa* (sand coprosma), *Pteridium esculentum* (bracken), and *Rubus fruiticosus* (blackberry). A total of 469 nests across both years were found in these substrates. The next vegetation type most frequently used was rushes, with 408 nests, mostly in *Apodasmia similis* (oioi) and *Ficinia nodosa* (knotted club-rush), occasionally in *Machaerina juncea* (tussock swamp twig rush) and various *Juncus* spp. The vegetation least frequently nested in were tall shrubs (>1 m); *Coprosma tenuicaulis* (hukihuki), *Coprosma robusta x propinqua* hybrids, *Kunzea* sp. (kānuka), *Plagianthus divaricatus* (makaka), and *Ozothamnus leptophyllus* (tauhinu), with only 125 nests found on these species. No nests were found in several habitat types: pasture grass and herb fields lacking woodier plants; permanently wet saltmarsh and dune lake areas (although nests were not uncommon on the periphery of more transitionally wet areas); under tree canopies; and sand pans and active sand dunes. Nests were also very rare in areas near (and especially downwind to the west of) active dunes, even where the habitat appeared otherwise favourable due to the presence of potential nesting sites.

In the 0.5 ha sample plots, the total number of nests found and recorded varied considerably, from $n = 4$ to $n = 93$ (Fig. 2.2). Within each plot, nests were often clustered in areas of what appeared to be favourable micro-habitat of landscape features and vegetation types. Plot 4 had a particularly high density of nests. This plot was close to a general area of high colony density, along the southern edge of the middle third of the spit (see Appendix C: Fig. C.3). This area is relatively sheltered from strong winds and has minimal influx from active sand dunes. The vegetation consists mostly of pohuehue, sand coprosma, bracken and blackberry, with patches of oioi, knotted club-rush, flax and tauhinu, and hardy pasture grasses,

herbs such as *Rumex acetosella* (sheep sorrel), and ground-covering *Leucopogon fraseri* (patotara) underfoot.

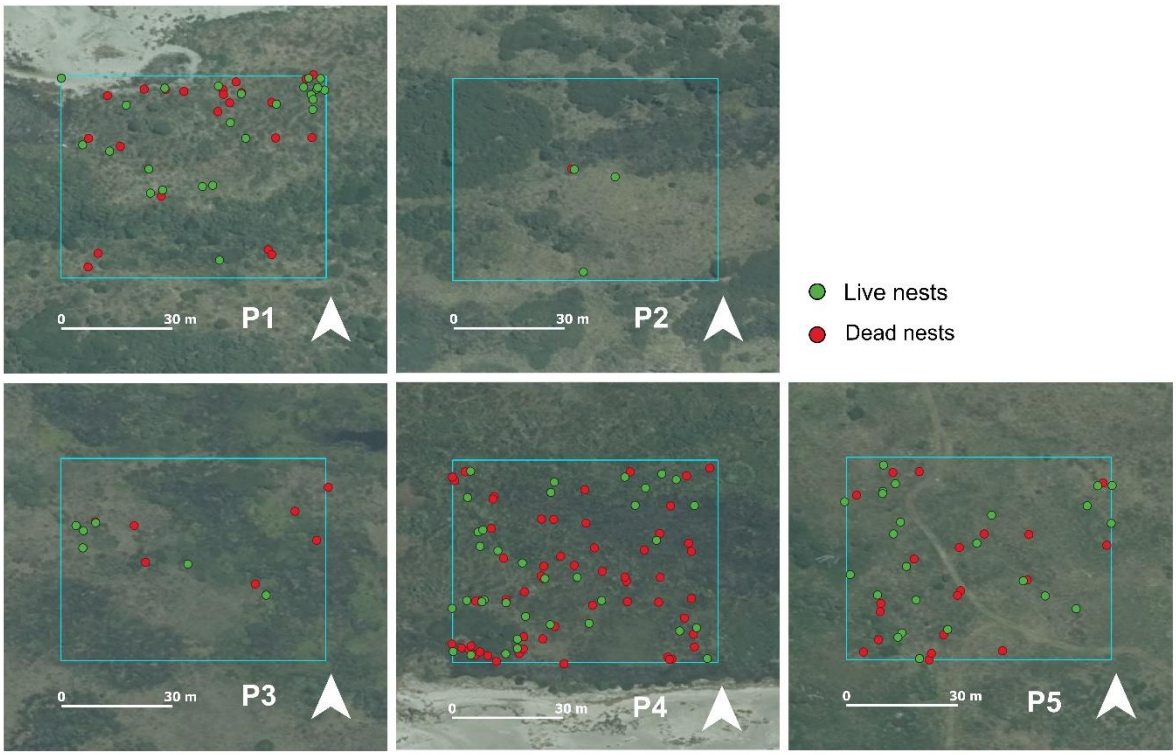


Figure 2.2 Sample plots 0.5 ha in area, randomly placed in QGIS 3.25, that were thoroughly searched for paper wasp nests. See Fig. 2.1 for location of plots. Green and red points mark each discovered nest, including nests that were dead or inactive at the time of discovery. Total nest counts for each plot were: P1 = 46, P2 = 4, P3 = 13, P4 = 93, P5 = 44. Nests show considerable clustering in some areas, usually falling along lines of landscape forms or in suitable habitat patches according to vegetation type or amount of sunlight. Aerial map imagery sourced from LINZ data service [accessed from https://data.linz.govt.nz/]. Map scale: 1:400.

Of the 77 nests established for monitoring in December 2020, 18.5% survived to the end of the monitoring period in February 2021. The survival rate of the 251 established nests in the next year was 22.2%, with 55 surviving colonies at the end of February 2022. The Kaplan-Meier survival analysis found no significant difference in the survival rates between these years (Fig. 2.3 B; log-rank test, $P = 0.501$). The overall survival curve for both years was similar, with the majority of colony mortality occurring in the early season (November and December) during the vulnerable founding and early superindividual stages.

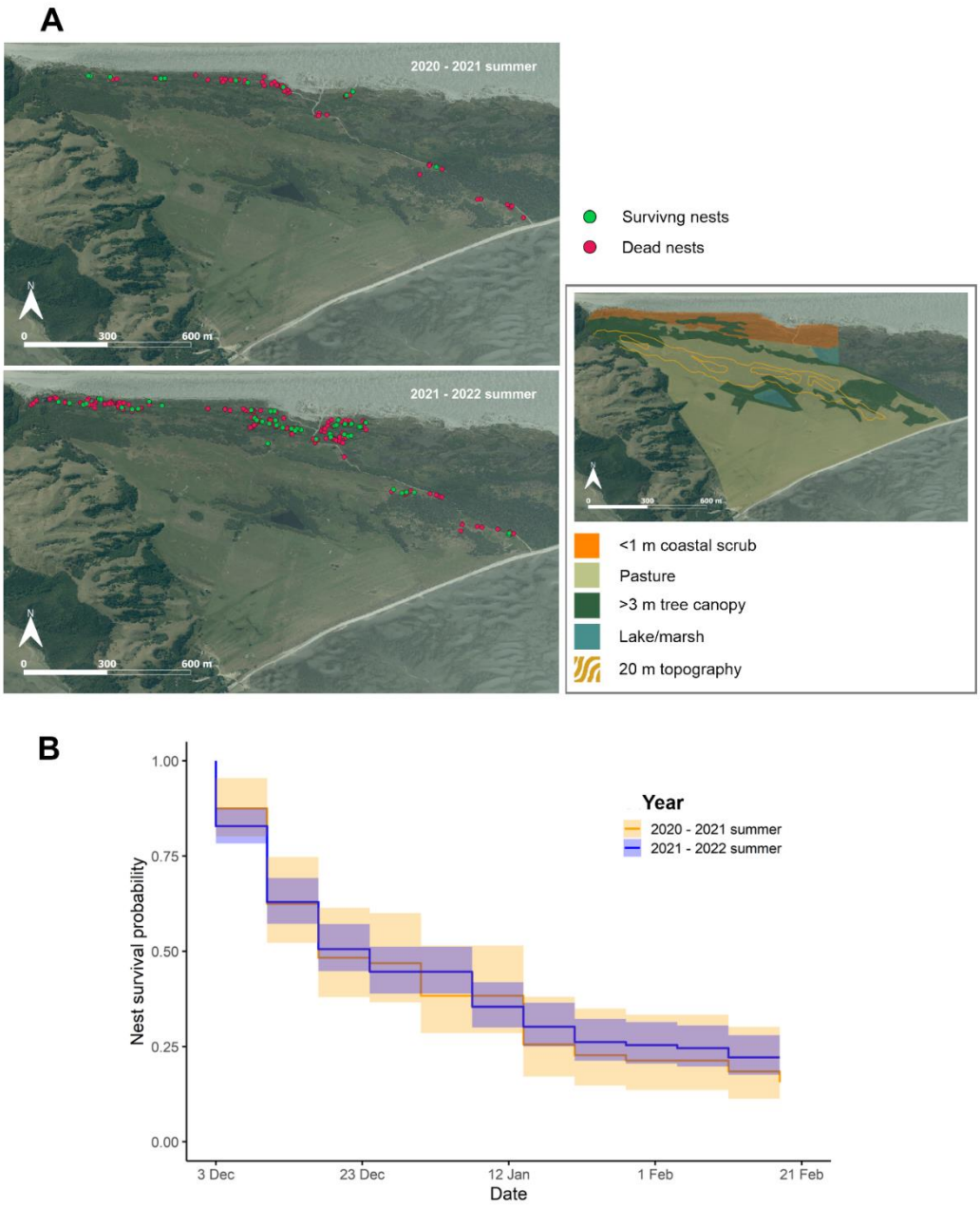


Figure 2.3 Paper wasp colony survival. **A)** GPS locations of nests monitored weekly in each study year of the 2020 – 2021 and 2021 – 2022 austral summers, within the approximately 120 ha study area at the eastern basal end of Onetahua. Green points are surviving nests in February of each year; red points are nests that did not survive. 2020 – 2021 season: *n* = 77; 2021 – 2022 season: *n* = 251. Alongside, the study area is mapped by habitat type and topography, showing nest clustering primarily in low elevation coastal scrub vegetation. Aerial imagery downloaded from https://data.linz.govt.nz/ and compiled in QGIS 3.25. Map scale: 1:4000. **B)** Kaplan-Meyer survival estimates (± 95% CI) for monitored colonies over time. Starting nests for years 1 and 2 were *n* = 77 and *n* = 251, respectively. A log-rank test was used to compare the survival rates of the monitored nests between years and found no significant difference (*P* = 0.501). The overall percentage of nests alive by the end of the monitoring period was approximately 20% in both years.

To attain an estimate of average nest density per hectare in late summer, I used the number of nests recorded from each 0.5 ha sample plot and values extracted from the survival analysis as outlined in Eq. 2.1. Each step of these calculations is presented in Table 2.1. The estimate of surviving colonies predicted per hectare based on each sample plot survey ranged from three to 54 colonies, with an average of 22.8. This result is based on numerous assumptions of survival across different habitat areas with potentially different time-varying factors, so should be considered an approximate estimate only, that may be of use for management purposes.

Table 2.1 Estimating the number of nests likely to survive to late summer in each sampling plot and producing a per-hectare average colony density, based on calculations from Equation 2.1. $\boldsymbol{n_{Total}}$ = number of wasp nests, including dead and uninhabited combs, found in each 0.5 ha plot; $\boldsymbol{n_{Alive}}$ = number of active wasp colonies found in each plot; $\boldsymbol{N_{Alive}}$ = active colonies per hectare ($2\boldsymbol{n_{Alive}}$); Date = date that plot was surveyed; $\boldsymbol{P_{Date}}$ = Kaplan-Meier survival probability of colonies at survey date; $\boldsymbol{N_{Start}}$ = estimated number of founded nests in late spring; $\boldsymbol{N_{End}}$ = projected number of active nests ha^{-1} in late summer.

Plot ID	n_{Total}	n_{Alive}	N_{Alive} (ha^{-1})	Date	P_{Date} (95% CI)	N_{Start} (95% CI; ha^{-1})	N_{End} (ha^{-1})
P1	46	30	60	12/12/21	0.63 (0.57, 0.69)	95 (87, 105)	21
P2	4	3	6	18/12/21	0.51 (0.45, 0.57)	12 (11,13)	3
P3	13	3	6	19/12/21	0.51 (0.45, 0.57)	12 (11,13)	3
P4	93	43	86	08/01/22	0.35 (0.30, 0.42)	246 (205, 287)	54
P5	44	27	54	13/01/22	0.35 (0.30, 0.42)	154 (129, 180)	34
						Average:	22.8

Nests developed at a similar rate over both early seasons, although more nests of the 2020-2021 summer remained in the founding phase into late January and February. As of 1 February 2021, 20% of nests had still not produced adults, compared to only 3% of nests at the same time in 2022 (Fig. 2.4). This variation is likely a result of climatic differences between years (Miyano, 1981). While there was no significant difference between colony demographics at the end of the monitoring periods in each year, differences in development rates such as this may result in inter-annual variation in the time at which wasp abundance peaks. Average cell counts, often used as an indicator of colony productivity, were 92.7 (±SE 15.7) and 107.5 (±SE 9.6) cells per nest in late-February nests in 2021 and 2022, respectively. Average numbers of adult wasps observed per nest were 8.6 (±SE 1.5) and 11.1 (±SE 1.0) in 2021 and 2022,

respectively. It should be noted that as nests were visited during the day when not all wasps will be on the nest, these averages should be considered conservative. The Wilcoxon rank-sum tests found these as well as other key colony demographics recorded to be non-significantly different between years (cells: $W = 266.5$, $P = 0.541$; larvae: $W = 368.0$, $P = 0.263$; pupae: $W = 346.5$, $P = 0.454$; adult wasps observed: $W = 247.0$, $P = 0.343$). The monitoring period for each year ended on 21 February, when most nests were in the superindividual phase, and had not yet produced males. Thus, total wasp abundance per colony likely would have continued to increase, potentially into March or April, before declining with colony dissolution (Clapperton & Dymock, 1997).

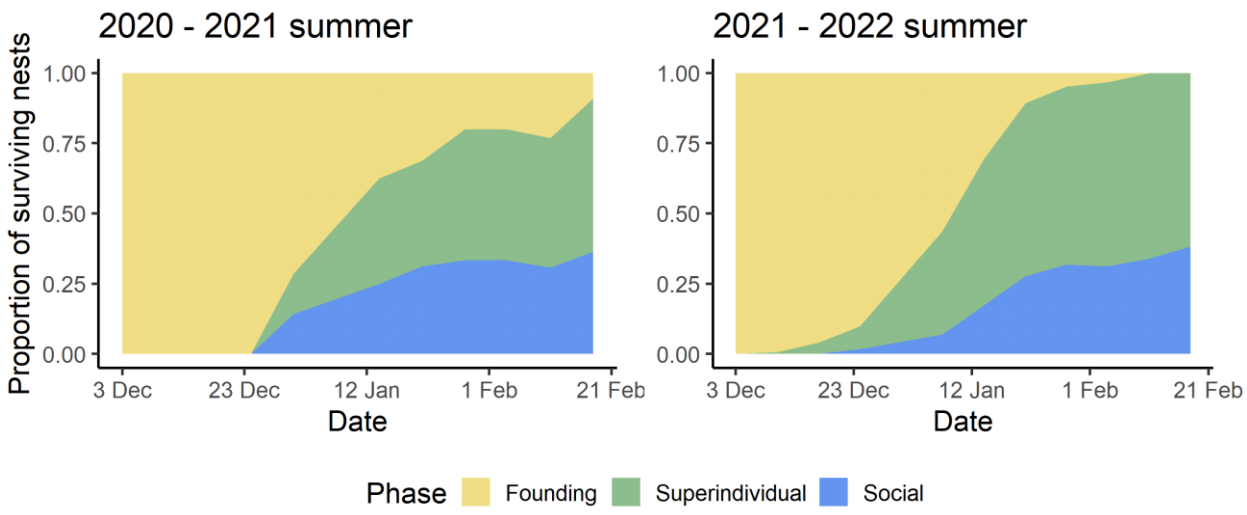


Figure 2.4 Area plots of the proportion of active nests in each phase of colony growth in each week of monitoring per year. Nests in the 'founding' phase had a foundress wasp and juveniles only; 'superindividual' nests had more than one adult female wasp; nests with one or more adult males were considered in the 'social' phase (Miyano, 1980; Sumner & Cini, 2021). As total wasp numbers per nest typically peak at the beginning of the social phase, these figures show that the wasp abundances observed at the end of the monitoring periods were unlikely to be peak wasp abundances for each year.

2.4.2 Field observations

Over the course of sample and data collection for this study, I observed foraging and hunting wasps and noted particular patterns of behaviour and plant and animal interactions whenever possible. I observed distinct behaviour patterns that appeared to be associated with the resource sought by each wasp. Wasps foraging for nectar resources spent more time crawling amongst vegetation to move from flower to flower, with occasional short flights to new vegetation patches. The plants I observed wasps to visit for nectar most frequently were pohuehue (*Muehlenbeckia complexa*; Fig. 2.5), followed by blackberry (*Rubus fruiticosa*), kānuka (*Kunzea* sp.), and bird's-foot trefoils (*Lotus* spp.). On one occasion a wasp was observed gathering fine, papery fibres from the dried flower stalk of harakeke plant (*Phormium tenax*) a few meters from her nest (Fig. 2.5). Wasps were frequently observed using their mandibles to "trim" encroaching twigs and other vegetation away from the immediate vicinity of their nest comb. Previously cut twigs were also often seen around a nest.

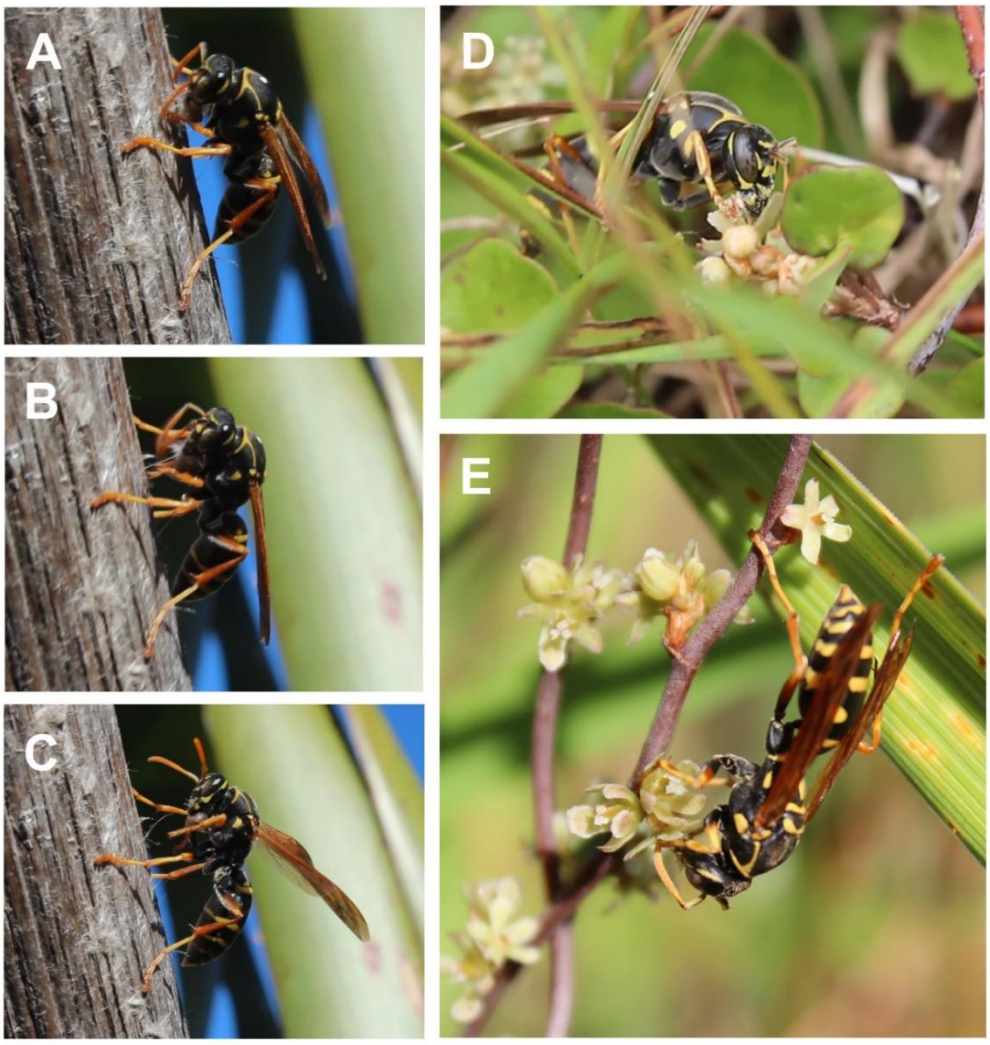


Figure 2.5 Foraging wasps collecting plant-based resources, observed on Onetahua (Farewell Spit) in December 2020 (A-C), and January (E) and November 2021 (D). **A-C)** A foundress wasp scraping fibres from the woody flower stem of a harakeke plant (*Phormium tenax*); **D,E)** Forager and foundress wasps gathering nectar from pohuehue (*Muehlenbeckia complexa*) flowers. For scale, the wasps are approximately 2 cm in length. Photographs: Aiden Reason.

In contrast to nectar foraging behaviour, hunting wasps displayed a methodical pattern of hovering flight over selective vegetation types, briefly alighting on or "striking" specific areas of a targeted plant. Pohuehue, kānuka, and blackberry were the plants most favoured for hunting prey as well as flower visiting. Young shoot tips were highly targeted areas, especially on pohuehue and kānuka. On blackberry brambles, furled or coiled leaves (current or abandoned caterpillar refuges) were frequently investigated. If caterpillar silk wrapping was found enclosed in a folded leaf, this was also torn apart with the mandibles. However, on most occasions an encased caterpillar was able to quickly wriggle free of the webbing and drop to safety, a behaviour which usually appeared to escape the wasps' notice. The main other plant type targeted for hunting were tall, erect grass and rush stems, especially flower or seed heads.

I observed wasps catching small, pale green or cream-coloured caterpillars from pohuehue plants on three occasions (Fig. 2.6 A), and from kānuka once. An *Uresiphita* sp. caterpillar was also seen captured from a lupin (*Lupinus arboreus*), and a small planthopper of a mottled grey colour was caught by a wasp from a blackberry stem. The exotic planthopper *Philaenus spumarius* (meadow froghopper) was also found in the diet analysis, a species that matches this description. One wasp was observed attacking a kēkēwai (blue damselfly, *Austrolestes colensonis*) recently caught in a spiderweb. The wasp cut away the damselfly's fleshy thorax, removing the wings and legs and leaving the abdomen and head, which it may or may not have returned for at a later time (Suzuki, 1978). On every occasion that I observed prey successfully caught and subdued, the body was quickly bundled into a compact mass, or in the case of larger prey, sliced apart with the wasps' mandibles before a chosen section was similarly bundled. Typically, the wasp would then make a short flight to a new location, before landing and methodically malaxating the bolus of flesh (Fig. 2.6 B). This process involved removal of presumably undesirable parts, such as fine hairs, sclerotised body parts such as the head, and sometimes gut matter. A return flight was then made to the nest, where the flesh ball was further malaxated and homogenised by the hunting wasp before feeding to larvae, or handed off to a nest-mate that would carry out this task instead (Fig. 2.6 C-E).

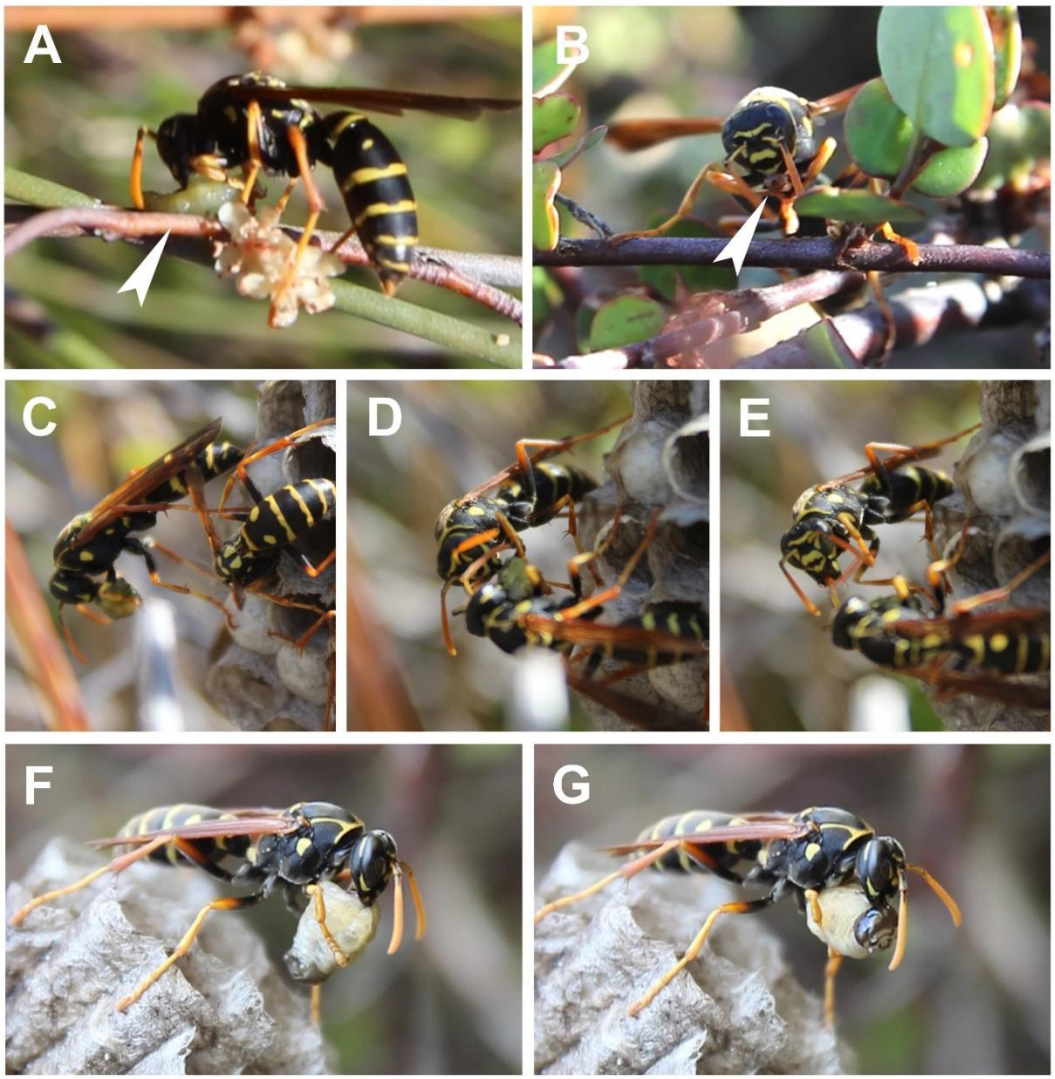
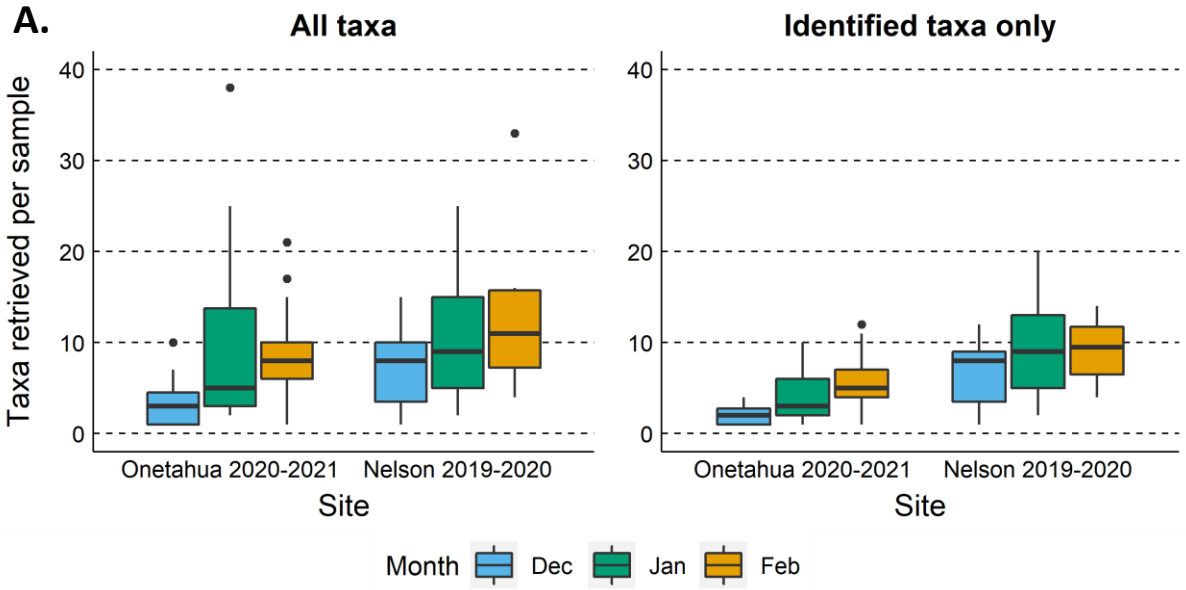


Figure 2.6 *Polistes chinensis* wasps catching and handling prey and flesh food. Photographs taken in the field on Farewell Spit, Nov 2020 – Feb 2021. **A)** A hunting wasp with a just-caught pale green caterpillar (arrow), immediately before taking off; **B)** A wasp holding a small prey bolus (arrow) with her forelegs and malaxating it away from the catch-site; **C-D)** A hunting wasp returning to the nest with a green prey bolus and handing it off to a nest mate for further processing and feeding to larvae; **F-G)** a foundress wasp consuming her own larva recently pulled from its nest cell. The entire body except the gut sac was consumed and redistributed to other larvae. For scale, the wasps are approximately 2 cm in length. Photographs: Aiden Reason.

2.4.3 Diet analysis

A total of 19,647,035 sequences were retrieved from the 72 Onetahua larva gut samples sequenced. Filtering for alignment length, percent identity, and a per-sample retrieval threshold, removed 11.1% of these sequences, leaving 17,467,539 reads. Removing sequences assigned to *Polistes* spp. left 3,981,156 reads (20.3%), sourced from 66 samples (Table A.1).

These sequences corresponded to 113 unique molecular operational taxonomic units (mOTUs) (Blaxter et al., 2005). Averaging across sampling times, 7.3 unique mOTUs were retrieved from each sample from Onetahua, with mOTUs per sample increasing over sampling times (Fig. 2.7 A). Ninety-one of these 113 mOTUs were identified to species and 22 identified to genus, with the reference sequence being taxonomically indeterminate. Out of 113 taxa, 91 belonged to the order Lepidoptera, nine to Hemiptera, five to Araneae, three Diptera, and one to Hymenoptera. There were also four non-target taxa: two plant species, *Fagopyrum esculentum* (buckwheat) and *Paederia foetida* (skunkvine, to current knowledge not present New Zealand (NZOR, 2012c)); a fungus *Puccinia graminis* (stem rust); and an oomycete *Peronospora polygoni.*



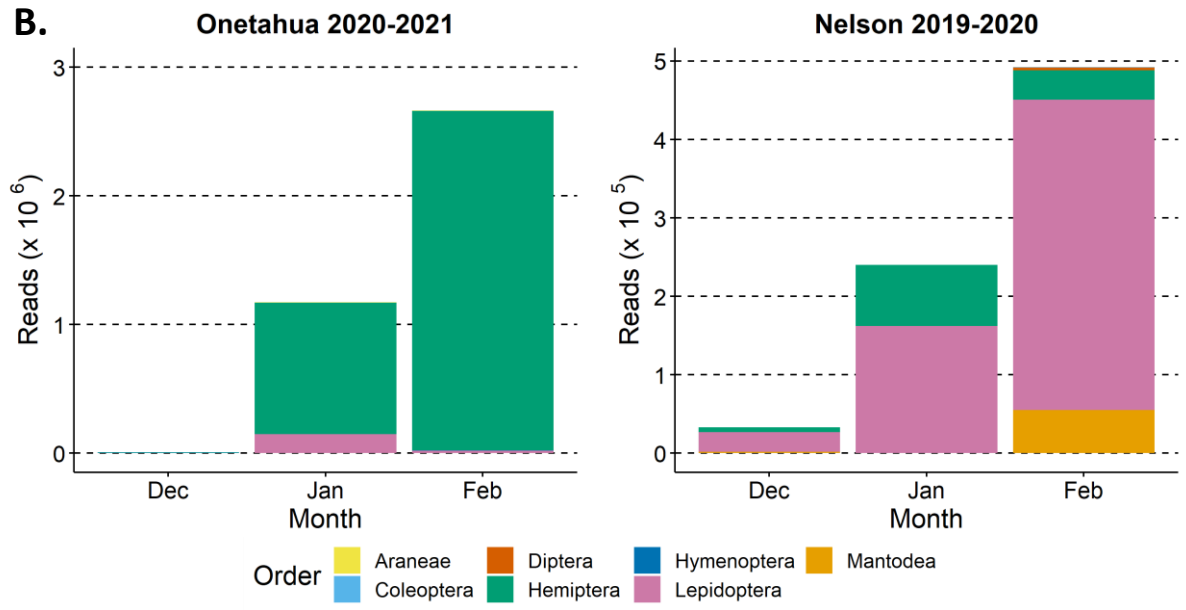


Figure 2.7 A breakdown of the taxonomic assignment for diet data from Onetahua, collected in this study, and a Nelson study, acquired from Howse et al. (2022). **A)** Boxplots for the average number of assigned taxa, or molecular operational taxonomic units (mOTUs), retrieved per sample from each sampling month in each site dataset, after data cleaning and removing *Polistes* reads. Onetahua *n* = 66; Nelson *n* = 29 (Table A.1). **B)** Read abundance per month for each sites' identified prey species, and corresponding taxonomic order, across all samples. See appendix A: Fig. A.1 for the same graph constructed using all assigned mOTUs. While read composition and proportion differs substantially between datasets, similar seasonal trends can be seen in both, despite reasonably balanced sample numbers across sampling months (Table A.1).

At both sites, read abundance differed substantially between sampling months, with the highest numbers retrieved from larvae taken in February (Fig. 2.7 B). The number of reads belonging to different Arthropod orders were highly disproportionate to relative species diversity in the Onetahua samples. Read abundance from all months at Onetahua was dominated by the order Hemiptera. Within these Hemiptera reads, 98.8% belonged to the family Cicadidae, and 95.6% to a single cicada species, *Rhodopsalta cruentata*, the endemic blood red-tail cicada. The much lower quantity of Lepidopteran reads from each sampling month corresponds to substantially greater species diversity (see Fig. 2.8). Read abundance and diversity were much more proportionate in the Nelson samples.

The Nelson dataset sourced from Howse et al. (2022) had a total of 14,187,711 sequences retrieved from 29 samples. The same filtering process removed 4.0% of reads due to sequence length, identity, or low read number per sample. Reads belonging to *Polistes* spp. constituted 94.6% of the total. After filtering, 770,785 sequences from 29 samples, corresponding to 87 unique mOTUs, remained. On average, 10.3 mOTUs were retrieved from each Nelson sample, also increasing over sampling times (Fig. 2.7 A). Seventy-one mOTUs had species-level identifications, and 16 were identified to genus but taxonomically indeterminate at species level. Overall, there were 74 Lepidoptera, six Hemiptera, two each of Araneae, Coleoptera, and Diptera, and one Mantodea species (Fig. 2.7 B). These results differ slightly from those reported in Howse et al. (2022) due to updates of the GenBank reference library that occurred after the library was accessed for taxonomic assignment in that study. This difference is most apparent with the addition of *Rhodopsalta* cicada barcode sequences to GenBank following a recent paper on the phylogeography of this genus by Bator et al. (2021).

After checking the assigned Arthropod taxa in each dataset against the NZOR and GBIF databases, I found that 63% of the Onetahua mOTUs, and 44% of those from Nelson, belonged

to genera or families recorded to be absent in Aotearoa. Therefore, these mOTUs could not be properly identified beyond family or order level, were classified as 'unidentified' species in further analyses, and assigned unique stand-in identifiers (for example: 'Geometridae 1'; 'Unknown family A sp. 3'). In both datasets, almost all unidentifiable mOTUs belonged to the order Lepidoptera (Fig. 2.8). I hypothesise that some of these taxa are likely to be rare species that have not been genetically sequenced and are not currently present in the reference GenBank database.

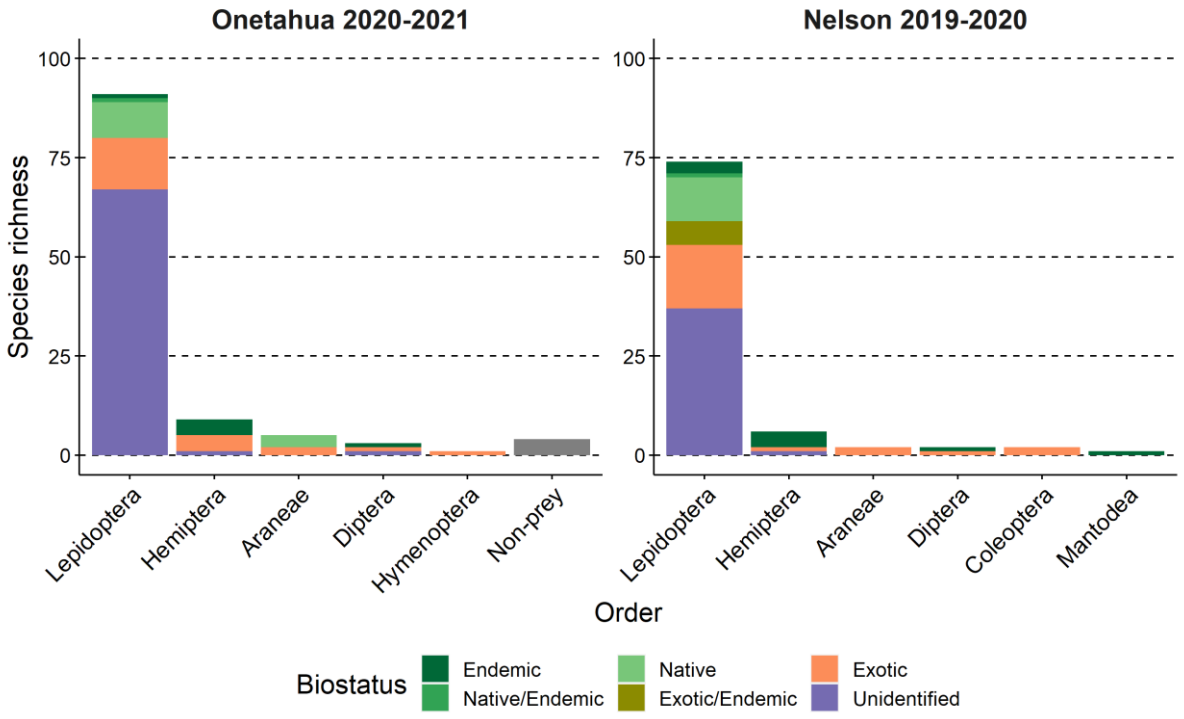


Figure 2.8 Number of species/mOTUs belonging to each Arthropod order from each sites' diet data, with corresponding occurrence and biostatus in New Zealand. Biostatus of the assigned species is represented here for those species known to be present in New Zealand. Due to a remaining level of uncertainty in taxonomic assignment, where the assigned species was indeterminate or is not known to be present in New Zealand, but congeneric species are present, the biostatus of those present congeneric species is represented. Where the genus (or, in eight cases, family) of an assigned taxon is absent from New Zealand, the mOTU was classified as 'unidentified.' Reference databases for occurrence and biostatus: GBIF, NZOR.

Of the 40 mOTUs from the Onetahua samples that were identified to species or genus level, six are endemic in New Zealand, 12 are native, and 21 are exotic. The remaining mOTU, which was identified to genus level only as the assigned species taxon was not present in New

Zealand, may be either a native or endemic species, as there are both native and endemic members of this genus in New Zealand.

The taxa retrieved most frequently from Onetahua samples were *Rhodopsalta cruentata* (Cicadidae; endemic), an unknown cicada species 'Cicadidae 1,' *R. leptomera* (Cicadidae; endemic), an unknown Noctuid moth, an *Agonopterix* sp. moth (Depressariidae; exotic), the parasitoid wasp *Meteorus pulchricornis* (Braconidae; exotic), and *Lycaena salustius* s.l. (Lycaenidae; endemic). Out of the 113 total mOTUs, 36 were present in ten or more samples (Fig. 2.9).

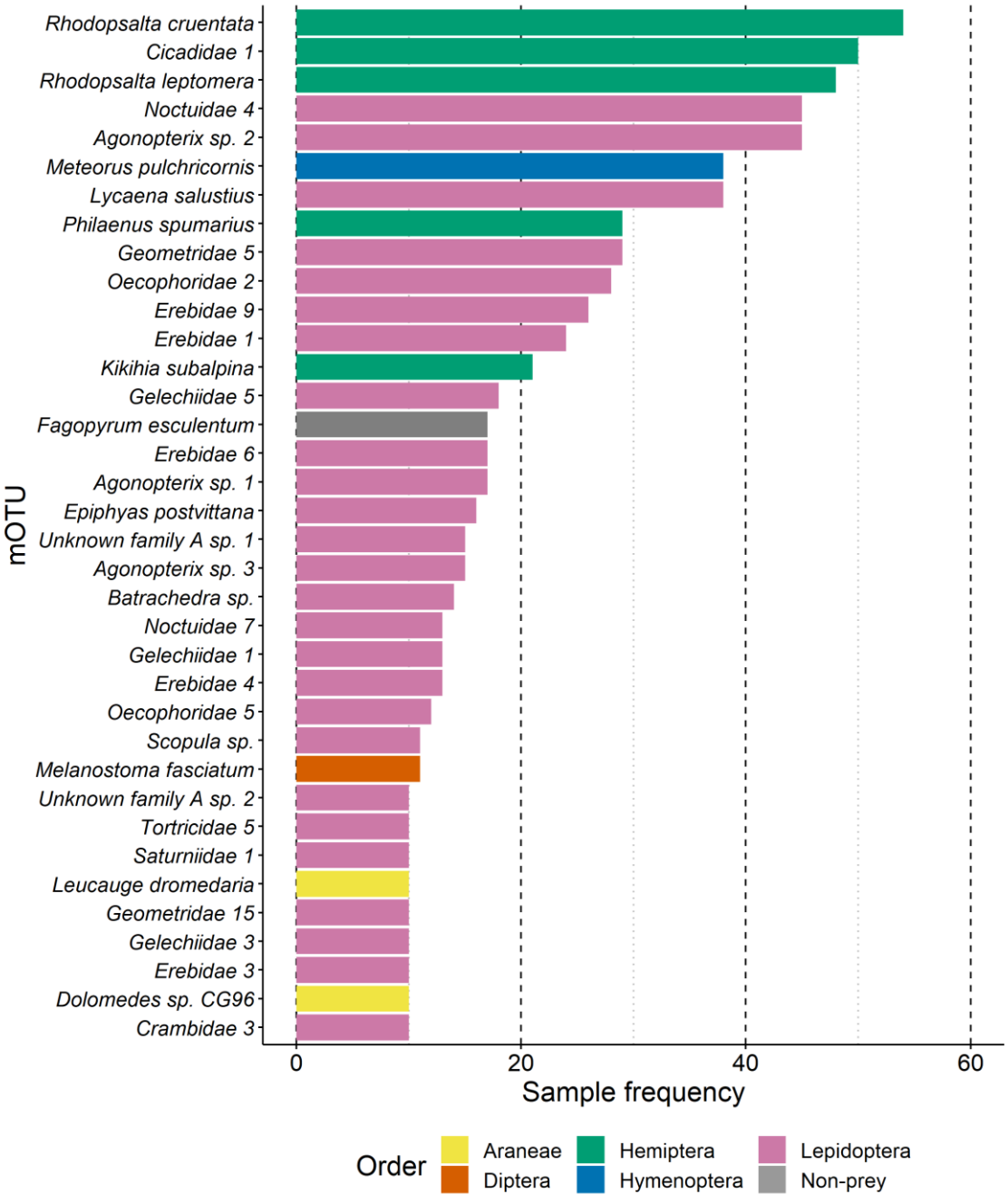


Figure 2.9 Unique species/mOTUs and the number of individual gut samples that their corresponding sequences were retrieved from, for mOTUs retrieved from ten or more samples only, also displaying taxonomic order.

There was an overall lower number of taxa that could be confidently identified found in Onetahua samples, despite having a larger number of gut samples than in the sampling from Nelson. This result is evident in the constructed species accumulation/rarefaction curves (Fig. 2.10). The upper end of the rarefaction curves from each sites' data remain relatively close together when all assigned mOTUs are included, but show greater disparity when the same curves are reconstructed including only taxa identified to genus or species level.

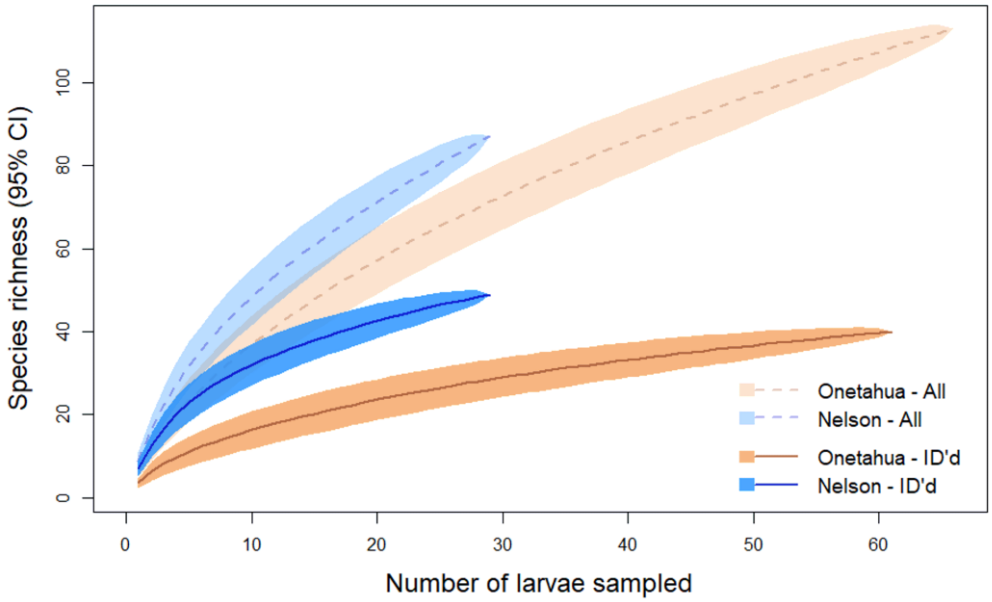


Figure 2.10 Species accumulation curves for each site, both for all mOTUs ("All") and identified Arthropod taxa only ("ID'd"). The rarefaction method was used to visualise diversity between samples as the number of 'new' mOTUs retrieved from each successive sample analysed. Eventual flattening of these curves would indicate completeness of the diet community sampled. The visualised curves do not completely flatten at the upper ends, indicating that a greater number of larvae samples taken would have increased the number of unique sequence matches retrieved.

Overall species richness and composition differed substantially between sampling months in the Onetahua dataset. The December samples had the lowest number of reads and lowest species richness. The greatest number of unique mOTUs retrieved were from January samples (Fig. 2.11). However, a large proportion of January mOTUs belonged to Lepidopteran groups not recorded from New Zealand (36 of 74 total; Table A.2). Excluding unidentified and non-target taxa, the greatest species richness then comes from February samples, including

four Hemipteran species appearing in no other group (*Kikihia muta, K. subalpina, Sidnia kinbergi,* and *Nezara viridula*).

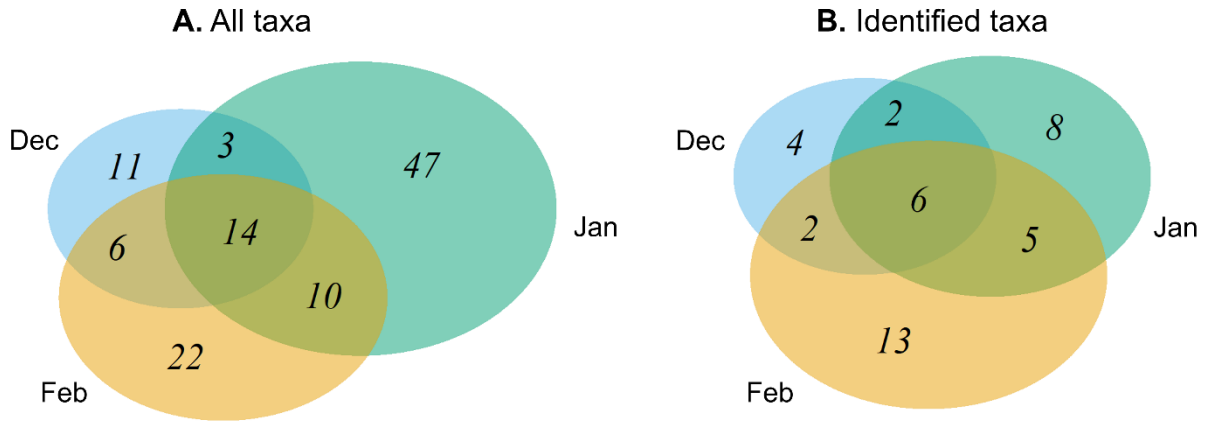


Figure 2.11 Venn diagrams of the number of species identified in the *Polistes chinensis* diet samples from each month of sampling from Onetahua only. **A)** Includes all assigned mOTUs; **B)** Includes only identified Arthropod taxa. See appendix A: Table A.2 for taxonomic lists in each month group, including intersectional groups.

2.5 Discussion

In this chapter I explored the colony survival rates and ecology of invasive paper wasps on Onetahua, determined the species composition of their prey, and identified prey species most likely to be threatened by wasp predation pressure based on colony development and frequency and timing of species consumption.

2.5.1 Colony and population dynamics

Across both nesting seasons, I found that *P. chinensis* nested predominantly in dry, low-growing scrub and rush vegetation, out of the range of influx from active sand dunes. As a result, the distribution of nests was patchy, with very low occupancy in some areas surveyed, and high densities in others, as has been previously observed by Ward and Morgan (2014). In late February of the 2021-2022 summer, the average number of adult wasps observed per nest was 11.1 (±SE 1.0), although this estimate should be considered a highly conservative number as nests were observed during the day when wasps from most nests would have been foraging. Extrapolations from survey plot and colony survival data gave a rough estimate for colony density at this time to be 22.8 colonies per hectare on average, a reasonable number given that 20-40 nests ha^{-1} are common in occupied areas (Ward & Morgan, 2014). These figures give us a

conservative, approximate estimate of 253 wasps ha^{-1} on average on Onetahua in late February 2022. This estimate makes an interesting comparison to the 154 wasps ha^{-1} found in Nelson coastal areas, in a similar study monitoring *P. chinensis* colony survival at the same time in 2020 (McGruddy, 2021). These two sites are relatively close geographically and have comparable climates, but likely have significant habitat differences due to the higher human presence in Nelson. The invasive congener *P. dominula* is also abundant around the Nelson region, with 256 wasps ha^{-1} in coastal areas sympatric with *P. chinensis* (McGruddy, 2021). *Polistes dominula* is a highly productive and adaptable species that has been shown to out-compete native *Polistes* species in *P. dominula*'s invaded range in North America and South Africa (Gamboa et al., 2004; Roets et al., 2019). Likewise, competitive exclusion of *P. chinensis* may be occurring where *P. dominula* has become established in Aotearoa. No individuals of *P. dominula* were observed in either year of data collection on Onetahua, although foraging wasps and nests of this species were found in the nearby townships of Onekaka, Bainham, and Collingwood, which are as close as 23 km away by road.

Colony survival rates were not significantly different between the 2020 – 2021 and 2021 – 2022 nesting seasons on Onetahua, with 18.5% of nests surviving the full monitoring period in the first year and 22.2% in the second year. These proportions are similar to those found in other studies, with a 22% survival rate reported in Auckland, 25% in Whangarei (Clapperton & Dymock, 1997), and 21% in Nelson coastal areas (McGruddy, 2021). Nest failure occurred at the highest rate in December, when over 90% of nests were in the founding phase, which is also consistent with previous research in Aotearoa (Clapperton & Dymock, 1997; McGruddy, 2021). This data confirms that this early stage of *P. chinensis* colony development is the most critical for ultimate colony survival. If a colony survives to mid-January it is very likely to survive the full nesting season and contribute to the high predation pressure exerted by the population in February.

Colony development timing did appear to differ slightly between monitoring years. I observed colonies developing more rapidly in the second monitoring year, with all monitored nests having produced adults by early February, compared to ~80% of nests the previous year. Varying rates of colony development have been previously observed across latitudinal gradients in Aotearoa, and is likely directly related to temperature and other climatic factors, possibly in combination with food availability (Clapperton & Dymock, 1997; Clapperton & Lo, 2000). Warmer temperatures are known to speed up brood development, and specific lower

temperature thresholds limit egg development and worker emergence in *Polistes* (Jeanne & Morgan, 1992; Miyano, 1981). Rainfall in November 2021 was approximately 54% the amount of November 2020, in the critical period of early development. January 2022 likewise had extremely low rainfall and was 1.4 C° warmer on average, likely offering greater potential for foraging during a phase of high nest growth (NIWA, 2022).

The proportion of active nests in the superindividual phase, characterised by the presence of non-reproductive daughter wasps, high population growth rates, and no males or gynes yet present (Miyano, 1980; Sumner & Cini, 2021), was greatest from late January onwards each year. The monitoring period of this study was limited to ending in late February each year, therefore I can make no estimates of the proportion of colonies in each phase past this time. However, this data coupled with previous research on the timespans of *Polistes* colony phases indicates that the abundance of hunting wasps (and thus exerted predation pressure) likely peaks in February (Clapperton & Dymock, 1997; Clapperton & Lo, 2000; Hoshikawa, 1981). Thus, prey species identified from February samples in the diet analysis are likely to be facing greater levels of wasp predation than species retrieved from December and January samples only, and may be more vulnerable to population-level impacts as a result.

2.5.2 Field observations

My observations of *P. chinensis'* hunting behaviours included a persistent pattern of wasps targeting plant parts most likely to be holding or concealing Lepidopteran caterpillars, such as soft plant shoot tips and rolled or damaged leaves. Richter (2000) also noted this behaviour in *Polybia* (Vespidae: Polistinae) foragers, which use visual cues of plant damage by leafroller caterpillars to locate prey. As visual hunters, *Polistes* wasps may be more likely to hunt conspicuous prey species (Richter, 2000). Conspicuous traits such as large spines or bright colours generally indicate mechanical or chemical defences against predation, and are often associated with phytophagous specialists (Macel, 2011). My observations of hunting wasps removing cuticular spines from caterpillar prey corroborate other research finding that *Polistes* are not deterred by such mechanical defences (Richter, 2000). The removal of the prey's gut, as I also observed, may be for the purposes of avoiding allelochemicals accumulated from the caterpillar's food plant – a feature often considered a chemical defence against predation (Richter, 2000).

2.5.3 Diet analysis

The diet analysis conducted using *P. chinensis* larvae gut samples returned results both corroborating and differing from similar studies on *P. chinensis* in New Zealand. The majority of species/mOTUs identified belonged to moths and butterflies (Lepidoptera), although 71% of these mOTUs were retrieved from less than ten samples, and 43% from less than five. The three mOTUs most ubiquitous across samples were all cicadas, with two species of *Rhodopsalta* cicada, and one unknown. Such a high proportion of cicadas in the prey community was somewhat unexpected, as *Polistes* are usually known as Lepidoptera specialists (Gould & Jeanne, 1984; Jeon et al., 2019; Kasper et al., 2004; Lefort et al., 2020; Ward & Ramón-Laca, 2013). However, they have also been found to be opportunistic predators in favour of foraging efficiency (Kudô, 1998). Cicadas have only been previously observed in the Aotearoa paper wasp prey community in Nelson, in which *Kikihia muta* was identified from 73% of *P. dominula* gut samples and 50% of those from *P. chinensis* (Howse et al., 2022). Geography may therefore have a significant impact these wasps' prey community composition. Variation from earlier analyses may also reflect updates to the GenBank database, such as the recent upload of *Rhodopsalta* barcodes from Bator et al. (2021). It should be noted that the ten most ubiquitous mOTUs were all retrieved from all three sampling times, with the exception of *Rhodopsalta leptomera* (in third place in order of sample frequency) which was retrieved only from January and February samples. Thus, mOTUs sampled in especially high frequencies may represent species whose lifecycle means they are present in the environment for a long period of the *Polistes* nesting season. Conversely, species identified from very few samples may have life cycles that make them vulnerable to *Polistes* predation for only a short period, are otherwise uncommon, or are not favoured prey species. Adults of *Rhodopsalta cruentata,* the most ubiquitous prey species, are typically active from November to March (Larivière et al., 2010; Logan & Connolly, 2005) and were frequently observed during data collection in the field.

While small in body size compared to Aotearoa's largest cicadas in the genus *Amphisalta*, *Rhodopsalta* and *Kikihia* cicadas are significantly larger in body mass than the average caterpillar, and no doubt make a substantial catch for a paper wasp. Thus, the issue of when and how *P. chinensis* hunters are able to capture these large prey items poses an interesting question. It may be the case that hunting wasps are able to capture and subdue these cicadas in the vulnerable stages during or soon after their emergence from the ground

and in their final moult, before their cuticle and wings have fully hardened. The identification of *R. leptomera*, the sand dune cicada, in Onetahua gut samples was a surprising result as this species is considered to be restricted to New Zealand's North Island (Bator et al., 2021).

Many species were retrieved from Onetahua samples that have also been identified in other diet studies, such as: *Uresiphita ornithopteralis*, *Poecilasthena pulchraria*, and *Lycaena* sp. (Lepidoptera) (Ward & Ramón-Laca, 2013); *Meteorus pulchricornis* (Hymenoptera) (Lefort et al., 2020); *Helicoverpa armigera* and *Scopula rubraria* (Lepidoptera) (Lefort et al., 2020; Ward & Ramón-Laca, 2013); *Chloroclystis filata*, *Epiphyas postvittana*, and *Lycaena salustius* s.l. (Lepidoptera) (Howse et al., 2022; Ward & Ramón-Laca, 2013); and *Melanostoma* sp. (Diptera) and *Holocola zopherana* (Lepidoptera) (Howse et al., 2022; Lefort et al., 2020). *Declana leptomera* and *Coleophora* sp. were found in all four studies (Howse et al., 2022; Lefort et al., 2020; Ward & Ramón-Laca, 2013). *Kikihia muta*, *K. subalpina*, *Rhodopsalta cruentata*, *R. leptomera*, *Philaenus spumarius* (Hemiptera), *Eudonia echo* (Lepidoptera), *Cryptachaea blattea* (Araneae), as well as one cicada and 12 Lepidoptera of various families that were unable to be identified, were all shared with Howse et al. (2022).

The Onetahua gut samples showed a similar ratio of species consumed from different Arthropod orders, and similar trends of read abundance per month of sampling, as the samples from Nelson. There was an overall greater prey species diversity found from Nelson for the number of samples, with more mOTUs retrieved from each sample on average, which could be due to a combination of several factors. Though sampling areas at Onetahua and Nelson spanned an overall similar area size, the Nelson site incorporated urban and suburban areas, with proximity to residential gardens, regenerative planting areas, and commercial horticulture (Howse et al., 2022). This highly heterogenous habitat likely supported a broader prey community, including both exotic and native species.

A substantial portion of both the Onetahua and Nelson prey communities consisted of mOTUs recorded to be absent from Aotearoa at the assigned taxonomic level. The total proportion of these was much higher in the Onetahua dataset however, with 63%. This result is most likely due to a relative lack of genetic studies on southern-hemisphere Arthropods, and thus a lack of sequence availability in the reference databases for these taxa (Meyer & Paulay, 2005; New & Samways, 2014; Nilsson et al., 2006). The higher proportion of these taxa assignments in the Onetahua data may be due to a bias in previous research efforts towards species naturally more common in urban and suburban areas, such as the Nelson area. Thus,

these taxa may represent a group of understudied, potentially highly endemic, or spatially localised species. Most unidentified taxa were from the samples collected in January, so it is possible that these may largely be species whose larval stage coincides with this time of year. The true taxonomic identity of these sequences remains unknown, but could be ascertained in future with dedicated taxonomic and sequencing work to improve the taxonomic completeness of genetic reference libraries.

The use of *Polistes* wasps as biological control agents for Lepidopterous agricultural pests has been previously explored (Southon et al., 2019). Indeed, the *P. chinensis* prey community from Onetahua included several exotic species with pest status, including some that have been targeted for biological control: *Helicoverpa armigera* (cotton bollworm), *Epiphyas postvittana* (light brown apple moth), *Coleophora spp.* (clover case-bearer moths), and *Nezara viridula* (green vegetable bug) (Cameron et al., 2006; Chynoweth et al., 2018; Ferguson et al., 2007; Rea et al., 2003; Suckling & Brockerhoff, 2010). There were also potentially three distinct species of *Agonopterix* moth present. Several species of this genus are themselves current or trialled biological control agents for three invasive plants in New Zealand: gorse (*Ulex europaeus*), common broom (*Cytisus scoparius*), and poison hemlock (*Conium maculatum*) (Chynoweth et al., 2018; Ferguson et al., 2007; Gourlay, 2021; Hoare, 2001; Suckling et al., 2000). Gorse is particularly abundant on Onetahua, likely due to the history of the site as cleared grazing land, and broom is also present though less abundant (Kelly, 1991; Tribe & Kennedy, 2010). Hemlock has not been recorded there and was not observed during data collection (Kelly, 1991). It is likely that the *Agonopterix* species sampled most frequently was *A. umbellana*, the gorse soft-shoot moth. It is possible that predation of this species may have negative effects on the impact *A. umbellana* is able to make as a biological control for gorse. Such cascading effects on plant productivity have been previously recorded as a result of paper wasp predation on monarch butterfly larvae (McGruddy et al., 2021b).

None of the species or mOTUs identified from Onetahua samples are currently listed as threatened in the New Zealand Threat Classification System (NZTCS, 2021). There is no direct evidence from this analysis to indicate that *P. chinensis* is impacting any specific taxa of conservation concern. However, congeneric species of several of the identified taxa are currently considered threatened (Hoare et al., 2017). As there is a reasonable potential for inaccuracy in sequence-based taxonomic assignment (Meyer & Paulay, 2005; Nilsson et al., 2006), the conservation status of species closely related to those identified is important to

consider. The assigned taxa *Lycaena salustius* and *L. bleusei* (‘*Lycaena* sp.’) warrant attention. Per Patrick and Patrick (2012), the *L. salustius* taxon includes the North Island population of coastal copper butterflies only, with South Island populations considered a complex of new *Lycaena* species. One such *Lycaena* sp., the ‘Nelson common copper’ was identified from Onetahua in 2019 (Patrick & Patrick, 2019). *Lycaena bleusei* is a species currently only known from Spain and Portugal (GBIF.org, 2022), thus may represent an uncertain *Lycaena* sp., potentially another undescribed ‘common copper’*,* or distinct New Zealand species. During the field study, copper butterflies matching the *Lyceana* common copper complex were frequently observed. Some individuals with wing markings typical of the type specimen for the *L. feredayi* complex, were also found (Gibbs, 1980a, 1980b) (Fig. A.2). There are two *Lycaena* species currently listed as ‘Nationally Critical’ in Aotearoa: *L. ianthina* and the taxonomically indeterminate boulder copper *Lycaena* sp. “Chrystall’s Beach” (Hoare et al., 2017). It is possible that other undescribed species may be at risk also.

The other two species identified with congenerics of conservation concern were *Declana leptomera* and an unknown *Pasiphila* sp. *Declana cf. hermione* is ‘Nationally critical,’ *Declana toreuta* and *Pasiphila* sp. “Olearia” ‘Nationally vulnerable,’ and *Declana griseata* and *Pasiphila sp. cf. magnimaculata* ‘Declining’ (Hoare et al., 2017). It should also be noted that the conservation status of many of the taxa identified in these diet analyses is unknown, as these populations have not been formally reviewed, or there is not sufficient data to make an informed assessment.

As seen from the monitoring of colony development in this study, predation pressure exerted by paper wasp colonies will likely peak each year from about late-January to February. Therefore, the prey species that are consumed during these times, whether due to prey species phenology or increased wasp hunting rates, may be the most vulnerable to negative population impacts from wasp predation. In both January and February, the endemic redtail cicadas *Rhodopsalta cruentata* and *R. leptomera* were the species identified from the most individual larval samples, along with an unknown species of cicada. Following these in abundance were *Lycaena salustius* (*Lycaena* sp. (Patrick & Patrick, 2012)) and *Agonopterix* “sp. 2” (Table 2.2). I would conclude that these species are likely to be bearing the highest impact of paper wasp predation on Onetahua. With present data, these species may be the most favourable to direct attention to in order to determine any population-level impacts of predation.

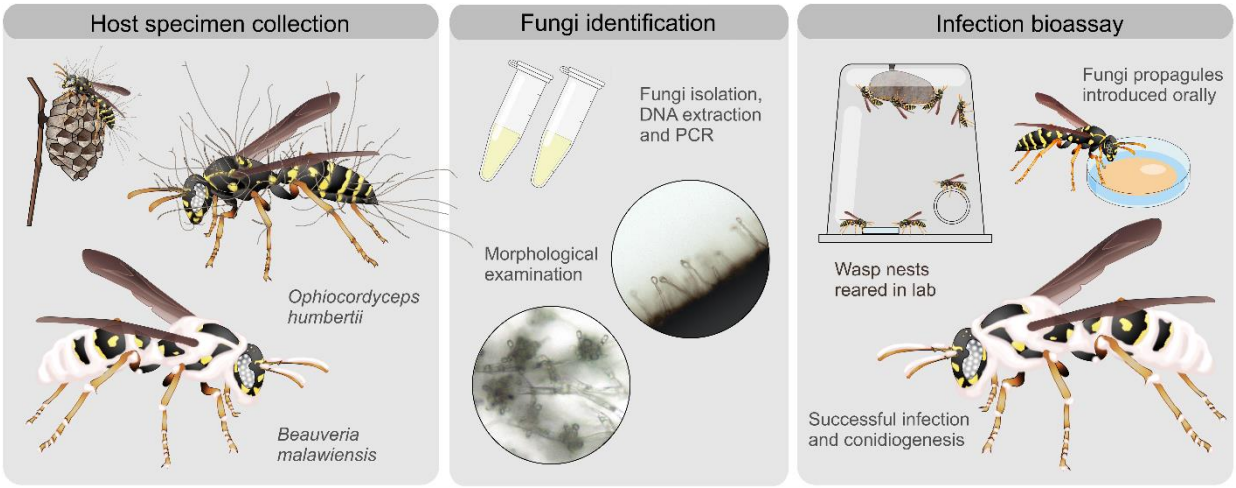
2.6 Conclusion

The average density of *P. chinensis* colonies surviving into late summer and the period of peak predation pressure on Onetahua, as estimated in this chapter, is typical for these wasps. My analysis of samples of the *P. chinensis* prey community also did not identify any taxa of published or otherwise obvious conservation concern. Therefore, these wasps might post no greater threat to invertebrates on Onetahua than in any other area of Aotearoa with similar colony densities. However, the high proportion of unidentifiable species in the prey community remains an unknown. If these taxa do represent an abundance of understudied, highly endemic, or cryptic species, potentially these species are more likely to include members with threatened or vulnerable populations. In the absence of a detailed genetic reference library for the rare and endangered species, this molecular approach may have limited use in identifying species of conservation concern. Invertebrate life cycles and phenology dependent on climate conditions are also worthy of consideration, as responses to changing climate conditions may alter *Polistes* prey community composition, shifting weight of predation pressure more or less on various species, or onto new species entirely.

Given the above, there is not enough evidence to suggest that there is an immediate necessity for control efforts on wasp numbers on Onetahua. Yet, the impressive diversity and abundance of terrestrial invertebrates in the Nature Reserve cannot be understated, and increased efforts to study and catalogue the insect community there would not be amiss. I recommend that annual monitoring of *Polistes* relative abundances be carried out to procure long-term data on the population size, and the same be done where possible for species identified in the prey community, such as the various cicada species and *Lycaena* sp. butterflies (see Appendix C). Such long-term population data is necessary to determine any population level impacts of predation on these species, as has been shown for butterfly populations under *P. dominula* predation pressure (McGruddy et al., 2021b).

Chapter 3: Identity, prevalence, and pathogenicity of entomopathogenic fungi infecting invasive *Polistes* (Vespidae: Polistinae) paper wasps in New Zealand

3.1 Abstract



Two species of entomogenous fungi were discovered infecting the invasive paper wasp *Polistes chinensis* during an ecological study on Farewell Spit, New Zealand. We sequenced two nuclear ribosomal DNA genes, the internal transcribed spacer (ITS) and the small ribosomal subunit 18S, and one protein-coding gene, the translation elongation factor 1-alpha (*ef1 α*). Combining sequence information with morphological examination, we identified these species as *Beauveria malawiensis* and *Ophiocordyceps humbertii*. We estimated that these fungi produce infection in approximately 3.3% of colonies in our study population. In bioassays, we successfully infected *P. chinensis* individuals from healthy colonies with *B. malawiensis*, with significant effects on adult mortality. This is the first record of both *B. malawiensis* and *O. humbertii* from Polistine hosts in New Zealand, and the first investigation into disease causality by these pathogens in *P. chinensis*. Our findings may contribute to future development of biological control agents for paper wasps in New Zealand, and elsewhere around the world.

3.2 Introduction

The concept of microbial pathogenicity and confirmation of disease causation, first introduced as Koch's postulates in 1855, has been adapted many times for application in various disciplines (Byrd & Segre, 2016; Evans, 1976; Fredericks & Relman, 1996; Hill, 1965; Inglis, 2007). The

establishment of causal links between fungal species and their infection of hosts is a relevant area of research for improved understanding of the evolution of microbial pathogenicity and host immunity (Joop & Vilcinskas, 2016; Lu & St. Leger, 2016; May & Anderson, 1983; Woolhouse et al., 2002), as well as for developing biological control methods for invasive species (Cummings, 2009; Harris et al., 2000; Mayorga-Ch et al., 2021; Mhlongwe, 2018; Rose et al., 1999). The pathogenicity of entomogenous fungi to social wasps has remained relatively unexplored compared to fungal associations with other insect groups, however, several studies have shown that there may be potential in these fungi for use in the control of pestiferous wasps (Harris et al., 2000; Mhlongwe, 2018; Rose et al., 1999).

Entomogenous fungi are important natural regulators of insect populations and are among the most abundant entomopathogens in the world (Goettel et al., 2005; Vega et al., 2012). They are a diverse group with members across four fungal phyla but are largely concentrated in the order Hypocreales of Ascomycota (Blackwell, 2010; Mora et al., 2018; Vega et al., 2012). Entomogenous fungi are unique as Arthropod pathogens in their infection mechanisms, typically entering their host through direct contact with the insect cuticle (Hajek, 2004; Inglis et al., 2001; Khachatourians & Qazi, 2008). These fungi produce specialised exocellular enzymes including proteases and chitinases that degrade the host cuticle, enabling the penetration of germ tubes or appressorial hyphae through the cuticle and epithelium (Boucias & Pendland, 1991; Inglis et al., 2001; Khachatourians & Qazi, 2008). Toxic secondary metabolites then facilitate invasion and colonisation of the host's haemolymph (Ortiz-Urquiza & Keyhani, 2013; Pedrini, 2018). However, several fungal species may also proliferate via the oral tract of the host if propagules are ingested (Harris et al., 2000; Mannino et al., 2019). There is ongoing interest in the research of fungal entomopathogens as biological control agents for Arthropod pests (Baker et al., 2020; Inglis et al., 2001; Jaronski, 2014). Several species, such as *Beauveria bassiana*, *B. brongniartii*, *Lecanicillium lecanii*, *Metarhizium anisopliae*, *Isaria fumosoroseus*, and *Hirsutella thompsonii* are increasingly economically important in commercially developed mycoinsecticides (Goettel et al., 2005; Jaronski, 2010, 2014; Kumar et al., 2019; Mascarin & Jaronski, 2016).

The Asian paper wasp *Polistes chinensis* is one of five eusocial wasp species considered invasive in New Zealand (Beggs et al., 2011; Howse et al., 2022; McGruddy et al., 2021a). It was accidentally introduced in 1979 and has since established a country-wide distribution (Clapperton et al., 1989; Clapperton et al., 1996). With no native representatives of Vespidae in

New Zealand, invasive eusocial wasps have little competition and have reached incredibly high densities in certain habitats (Lester et al., 2013; Thomas et al., 1990). These omnivorous wasps have catastrophic impacts on native invertebrate communities, and further disrupt ecosystems by competing for carbohydrate resources (Beggs & Jo, 1999; Beggs & Wardle, 2006; Toft & Rees, 1998). *Polistes* wasps in particular have been found to cause significant declines in butterfly populations, which can have cascading effects on plant productivity (McGruddy et al., 2021b). There is currently no efficient method for the control of paper wasp populations, and they are not attracted to the protein baits used for invasive *Vespula* wasps in New Zealand (Beggs et al., 2011; Toft & Harris, 2004). Options for biological control agents for *Polistes* species are currently the most promising avenue of research in this area (Brown, 2021).

During an ecological study of invasive *Polistes* paper wasps on Farewell Spit in New Zealand's South Island, two morphologically distinct species of entomopathogenic fungi were discovered parasitising *Polistes chinensis.* The coastal saline vegetation abundant in this site has been identified as a key habitat area in which to study the ecological impacts of *Polistes chinensis* in New Zealand, as colonies reach the highest recorded densities in these habitats (Ward & Morgan, 2014). The site is also a national Nature Reserve (Government, 1977) and Ramsar Wetland site (Ramsar, 1971), recently recognised for housing an impressive invertebrate diversity, including several rare and threatened species (Patrick & Patrick, 2019; Toft, 2020; Trewick & Morgan-Richards, 2020).

The aims of our study were: 1) to identify the two fungi morphospecies found infecting *Polistes chinensis* by combining genetic and morphological methods; 2) to test for the field prevalence of *Beauveria* sp. in samples of *P. chinensis* adults and larvae collected from wild nests; and 3) to test for disease causation in *P. chinensis* using samples of the identified fungi under controlled laboratory conditions.

3.3 Materials and methods

3.3.1 Study site, sample and nest collection

Field work was conducted on Farewell Spit, New Zealand (40.513°S, 172.852°E) during the austral summer 2020 – 2021 and 2021 – 2022 seasons. Farewell Spit is a 25 km-long barrier sand spit extending east from the northern headland of New Zealand's South Island (Tribe & Kennedy, 2010). It encompasses active and stable sand dunes, saltmarsh, and coastal scrub

habitats (Kelly, 1991; Petyt, 1999). The climate is temperate marine, with high relative humidity (yearly average 80.7%) and strong winds (NIWA, 2022).

Locations of *Polistes chinensis* nests were established after visual searches in late November of 2020 and 2021 and monitored weekly through to late February of 2021 and 2022, respectively. With only three *Polistes* species currently present in New Zealand, the wasps were easily differentiated from congeneric species *P. dominula* and *P. humilis* by their colouration, particularly of the scutellum and second abdominal tergite, and patterns on the scutum (Clapperton et al., 1989; Clapperton et al., 1996; M.P.I, 2016). Nests were primarily attached to low-growing, twiggy vegetation and rushes, the most common plant species being *Muehlenbeckia complexa*, *Coprosma acerosa*, *Ficinia nodosa*, *Coprosma tenuicaulis*, *Apodasmia similis*, and *Pteridium esculentum*, in order of frequency.

For molecular and morphological identification of fungi species, we collected dead and mycosed adult wasps on their nest combs (*n* = 17; Table B.1). For analysis of fungi prevalence in the wasp population we collected healthy larvae (*n* = 12 nests) and adults (*n* = 8 nests) from monitored nests, with two individuals of each life stage from each nest. In the latter case, neither the larvae, the adults, nor their nests of origin showed signs of disease. All samples were either frozen or collected in 100% ethanol in the field and stored at -80°C once in the laboratory.

For the laboratory infection bioassay, ten whole paper wasp nests were collected in mid-February of 2021 during daylight hours. Three of these nests had dead foundresses with presumed fungal infection (see section 3.4.2 and Table B.1) affixed to the nest, as well as live adults. In two of these afflicted nests the emerged adult wasps had continued to construct the nest comb around the body of the foundress, so that it was partially covered in paper. The remaining seven nests were visually healthy, and were used in bioassay treatment and control groups. All nests were extracted by cutting away a small amount of the substrate vegetation and enclosing them in a ventilated plastic container. We remained at the nest sites up to half an hour after removing the nests to collect any returning adults.

3.3.2 Fungal identification

DNA extraction, sequencing and phylogenetics

We used four mycosed *P. chinensis* adult individuals for genetic identification of the infecting fungi, two per fungal species. For each fungal species, one sample was harvested from the

outside of a frozen wasp cadaver, and one sample was harvested from the outside of a wasp cadaver stored in 100% ethanol. For each sample, we harvested 100 mg of conidia or fungal growth. The starting fungal material was collected in a 2 mL microtube (Sarstedt, Germany). We added three 3.2 mm stainless steel beads (Next Advance Inc., USA), 1 mL of GENEzol DNA Plant Reagent (Geneaid Biotech, Taiwan) and 5 µL of β-mercaptoethanol (Sigma Aldrich, USA) to the tube. Samples were homogenised for two cycles of 10 s each at 10,000 rpm in a Precellys Evolution homogeniser (Bertin, France). We used a 24:1 chloroform–isoamyl alcohol mixture (BioUltra, Sigma Aldrich, USA) to isolate DNA, followed by isopropanol precipitation (BioReagent, Sigma Aldrich, USA), and a 70% ethanol purification step (VWR Chemicals, UK). Lastly, DNA was eluted in 75 µL of nuclease-free water (Ambion, Life Technologies, USA) and quantified using a NP80 NanoPhotometer (Implen, Germany).

Initially, we amplified two commonly used nuclear biomarkers for fungal identification, ribosomal DNA internal transcribed spacer (ITS) and ribosomal DNA small subunit 18S (Badotti et al., 2018; Schoch et al., 2012). Primer pairs used were ITS1–ITS4 for ITS (Forward primer ITS4: 5'-TCCTCCGCTTATTGATATGC-3' and reverse primer ITS1: 5'-TCCGTAGGTGAACCTGCGG-3') and NS1–NS6 for 18S (Forward primer NS6: 5'-GCATCACAGACCTGTTATTGCCTC-3' and reverse primer NS1: 5'-GTAGTCATATGCTTGTCTC-3') from White et al. (1990). After the initial screening, one of the fungal species was identified as *Beauveria* sp. Thus, we also amplified the translation elongation factor 1-alpha gene (*ef1* α) using primers developed for *Beauveria* species identification in New Zealand by McKinnon et al. (2018) (see section 3.3.3 below for primer sequences and further details). The reaction volume was 15 µL containing 1x MyTaq Red Mix (Bioline, Australia), 5 pmol of each primer, bovine albumin serum, fungal DNA, and nuclease-free water. Non-template controls were included in each PCR. We visualised PCR products by 2% agarose gel electrophoresis. Most PCR products were purified with rSap combined with Exo 1 (New England Biolabs, USA). The *ef1* α gene showed multiple bands, so the amplicon of the desired length was excised from the gel and purified with Zymoclean Gel DNA Recovery Kit (Zymo Research, USA). Sequencing was performed on an ABI 3130x1 Genetic Analyzer (Applied Biosystems, USA) at Massey Genome Service (Palmerston North, New Zealand).

Chromatograms were visualised, manually inspected for ambiguities and aligned using Geneious v. 10.2.6 (http://www.geneious.com, Kearse et al., 2012) with the default Geneious alignment algorithm. We manually trimmed the low quality base calls at each end. We then blasted our fungal sequences into the BLASTn database in the NCBI website (Altschul et al.,

1990). We searched against the rRNA/ITS databases. For ITS, we specifically searched the internal transcribed spacer region (ITS) from Fungi type and reference material. For 18S, we searched the 18S ribosomal RNA sequences (SSU) from Fungi type and reference material. For *ef1* α, we searched the BLASTn standard database (default option). All sequences are archived in GenBank (accession numbers are presented in Table 3.1).

Table 3.1 GenBank accession numbers for each fungal isolate sampled from four *Polistes chinensis* wasp hosts. We amplified three regions for the *Beauveria* species, identified as *B. malawiensis*: internal transcribed spacer (ITS), small ribosomal subunit 18S, and translation elongation factor 1-alpha (*ef1* α). For the hirsutelloid species, identified as *Ophiocordyceps humbertii*, we amplified only ITS and 18S, as the elongation factor primers are *Beauveria*-specific.

Gene	Primer ref.	Fungal species	Lab ID	GenBank accession no.
ITS	(White et al., 1990)	*O. humbertii*	1	ON479659
			2	ON479660
		B. malawiensis	3	Not submitted
			4	OL347578
18S	(White et al., 1990)	*O. humbertii*	1	ON458754
			2	ON458755
		B. malawiensis	3	OL336513
			4	OL336514
ef1 a	(McKinnon et al., 2018)	*B. malawiensis*	3	OL348210
			4	OL348211

To construct a phylogenetic tree for *Beauveria*, we downloaded the ITS sequences for the *Beauveria* species presented in Imoulan *et al*. (Imoulan et al., 2016) (GenBank accession numbers listed in Table 1 in Imoulan *et al*. (Imoulan et al., 2016)). An ITS sequence for *Cordyceps militaris* was also included as the outgroup. Sequences were aligned with Geneious with the default alignment algorithm, using global alignment with free ends and gaps and a cost matrix equal to 70% similarity. Clade probabilities were obtained from the posterior distribution using the MrBayes v.3.2.6 plug-in (Huelsenbeck & Ronquist, 2001) for Geneious. Bayesian analyses were replicated twice, each with four Markov chains of 5 million generations, using a time-reversible model and gamma-distributed rate variation, with a proportion of

invariant sites. Trees were sampled every 2,500 generations, of which the first 150,000 generations were discarded as burn-in.

Morphological characterisation

At the macroscopic level, the first fungal species had a pale, powdery appearance characteristic of the white muscardine disease caused by *Beauveria* species (Kumar et al., 1999), emerging from between segments and joints in the wasps' cuticle (Fig. 3.1). Mycosed wasp specimens infected with the other fungus species developed white to brown, filamentous synnemata, emerging from the hosts' intersegmental membranes (Fig. 3.2), similar to descriptions for hirsutelloid *Ophiocordyceps* species (Hall et al., 2012; Toledo et al., 2013).

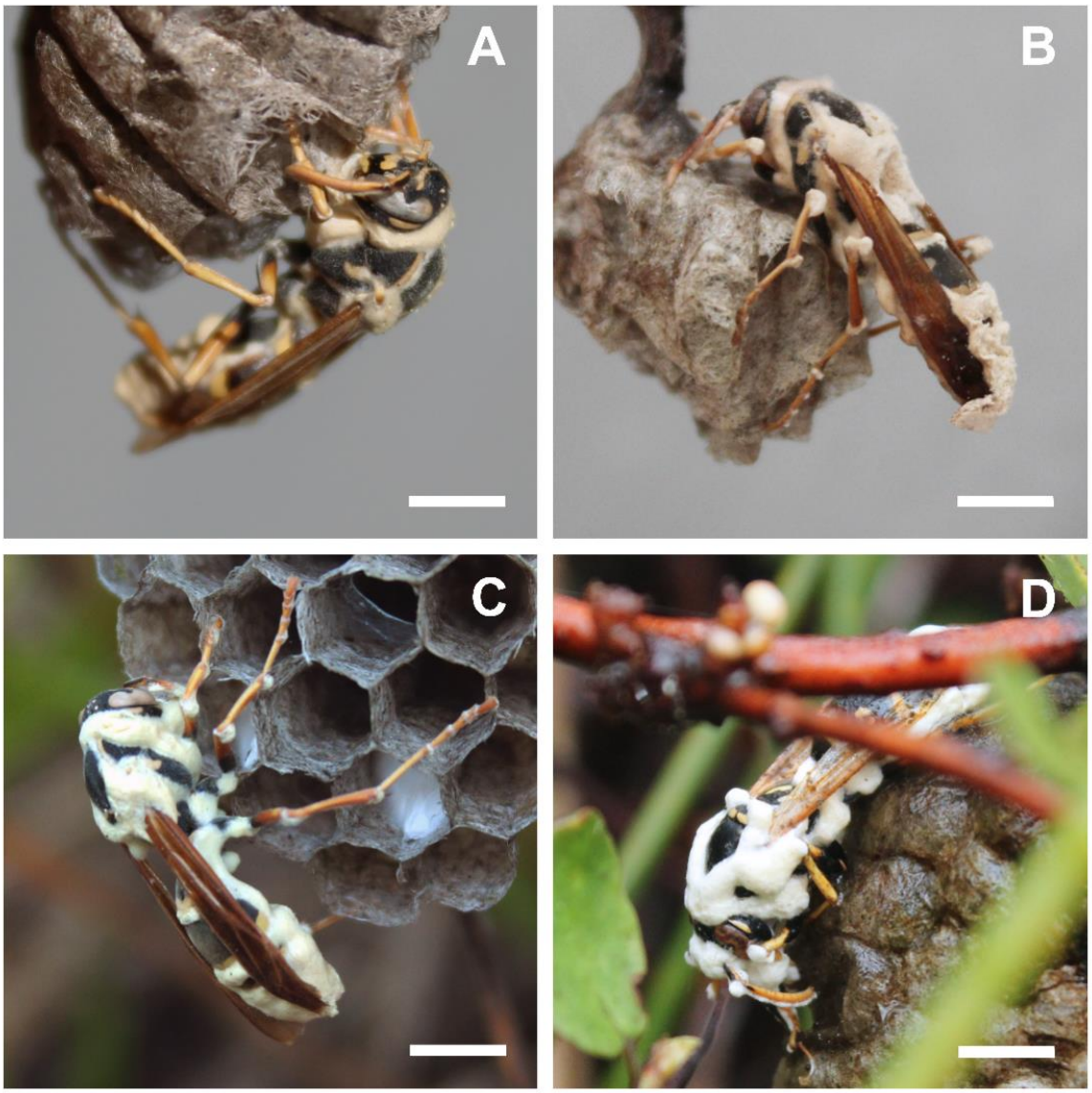


Figure 3.1 *Polistes chinensis* wasps infected with entomogenous fungus identified in this study as *Beauveria malawiensis*. These photos show host specimens on founding-phase combs with no brood, although host nests were found in both founding and superindividual phases. **A,B)** Mycosed wasp cadavers on nest combs collected from the field on Farewell Spit, Dec 2020 and Jan 2021, respectively, photographs taken in the laboratory. **C,D)** Mycosed wasp cadavers on nest combs *in situ* on Farewell Spit, Jan 2021 and Feb 2022, respectively. These specimens were discovered and collected at a mature stage of conidiogenesis, with significant external fungal bodies visible. Slight pinkish colouration of the fungal growth is evident in photo B, specifically on the antennae. Scale bars = ~0.5 cm. Photographs: Aiden Reason.

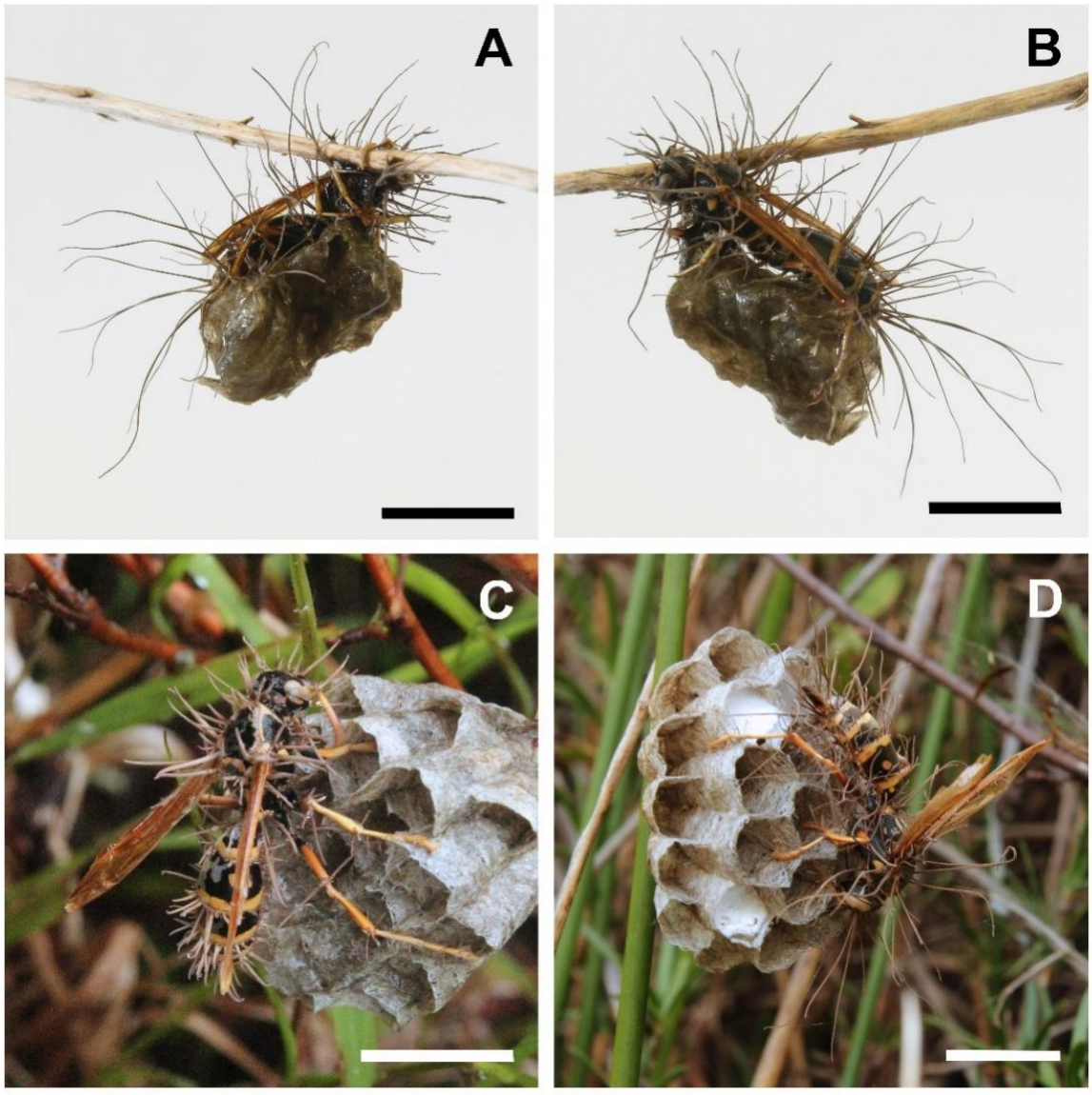


Figure 3.2 *Polistes chinensis* wasps infected with entomogenous fungus identified in this study as *Ophiocordyceps humbertii*. All specimens of this species were found on *P. chinensis* hosts affixed by their legs to the nest combs. These photos show host specimens on founding-phase combs with no brood, although host nests were found in both founding and superindividual phases. **A,B)** A mycosed wasp cadaver on a waterlogged nest comb, collected from the field on Farewell Spit in Dec 2021, photograph

taken in the laboratory. **C,D)** Mycosed wasp cadavers on nest combs *in situ* on Farewell Spit, Dec 2021 and Jan 2022, respectively. Scale bars = ~1.0 cm. Photographs: Aiden Reason.

For examination of fungal microscopic morphology, fungal samples were harvested from wasp cadavers under a dissecting microscope and mounted directly to microscope slides (Inglis et al., 2012). An Olympus CX41RF compound microscope with an Olympus DP22 digital camera was used to photograph and visually analyse samples.

3.3.3 Prevalence of fungi in wild nests

We used the collected samples of individual adult wasps and larvae to test for presence of entomogenous fungi in apparently healthy individuals. Each larva was dissected, and their gut sac removed. The body remains were used for DNA extraction, for a total of 24 larvae from twelve different nests. We also extracted DNA from sixteen adult wasps from eight nests. DNA extraction followed the same protocols as described in section 3.3.2 for molecular fungi identification.

To determine presence/absence of *Beauveria* spp. in these samples, we followed the nested PCR approach for identifying *Beauveria* species in New Zealand plants developed by McKinnon *et al.* (2018). Two-step nested PCR consists of standard PCR with primer pair 1, followed by standard PCR with primer pair 2. Briefly, a first primer pair, EF3F (5'-ACGGTGCCCGTCGGT-3') and EF5R (5'-ACTTGATGAACTTGGGGTTGTTC-3'), amplifies 406 base-pairs (bp) of the *ef1* α gene from multiple species of *Beauveria.* A second primer pair, EF4F (5'-GTCGCTGGTGACTCCAAGAA-3') and EF4R (5'-GTACGGCGGTCGATCTTCTC-3'), amplifies a shorter fragment of the gene of approximately 200 bp, nested within the previous amplicon, and the resulting sequence contains positions where single nucleotide polymorphisms enable species identification (McKinnon et al., 2018). The PCR reaction volume was 15 µL containing 1x MyTaq Red Mix, 5 pmol of each primer, bovine albumin serum, nuclease-free water, and 2 µL of wasp DNA (1 µL from each individual from the same nest). Non-template controls consisting of all the above reagents minus the DNA, and two independent samples positive for *Beauveria* extracted from infected adult *P. chinensis* wasps were included in each amplification assay. All PCR products were visualised by 2% agarose gel electrophoresis.

3.3.4 Infection bioassays

Nests collected from the field were transported to the laboratory and transferred from collection containers into ventilated 9 L plastic, transparent boxes. Combs were secured by their supporting plant material to the box ceilings with wire. Eight nest boxes were used in total, with seven having one nest per box, and one with three nests in one "group" box. Three of the ten collected nests had a dead foundress affixed to the comb, presumably killed by fungal infection, but without evidence of conidiogenesis. Two of these infected nests were placed together with a third, apparently healthy nest, in the group box. The last infected nest was placed in its own box. These two nest boxes containing nests with infected foundresses were maintained free of bioassay manipulations solely for the purpose of monitoring the potential for infected foundresses cadavers to undergo conidiogenesis under increased humidity conditions, and potentially infect other wasps (Goettel et al., 2005; Inglis et al., 2012). All the nest boxes were kept in a temperature-controlled rearing room set to 23.0 °C (varying with manual humidity changes) with an automated day-night light cycle of 12 hours. Relative humidity was manually manipulated with five large water trays on elevated shelving and the use of two electric humidifiers (3.7 L and 5.6 L; Anko brand, New Zealand), which were refilled daily. Both temperature and relative humidity were automatically logged hourly. Forager wasps were supplied with 30% sugar water, fresh water, cardboard paper for nest building, and wax moth larvae (*Galleria mellonella*) *ad libitum*. All wasps were allowed to acclimate for two weeks in the rearing room, during which time the average temperature was 23.8 °C (±SE 0.1), and the average relative humidity was 47.1 % (±SE 0.8).

A conidial suspension was prepared for each fungal species using the isolates harvested from the outside of host cadavers collected in the field. The conidia of the presumed *Beauveria* sp. were found to be highly hydrophobic and so were suspended in a solution of water with 0.05% Triton X-100 buffer (BioUltra, Merck, New Zealand) (Harris et al., 2000) to improve suspension homogeneity. The concentration of conidia in this solution was measured using a Neubauer haemocytometer and determined to be 2.68 $x10^6$ cfu (colony forming units) mL^{-1} (Inglis et al., 2012). The synnemata isolated from the hirsutelloid fungus were suspended in water only, and the mixture gently agitated to release conidia. The conidia concentration of this solution was 1.52 $x10^6$ cfu mL^{-1}.

In a pilot experiment, we attempted host wasp infection using a spray method of inoculation with the conidia suspensions (and control solutions without conidia) as implemented in Inglis et al. (2012), but it did not result in any observable infectious effect. Thus, in this study, infection by oral route was attempted following methods outlined in Harris et al. (2000). The conidia suspensions were mixed 50:50 with 30% sugar water to be fed to treatment nests (n = 3). Similar solutions of 50:50 sugar water and the appropriate carrier per fungi treatment (pure water or water with 0.05% Triton X-100 buffer) were supplied to control nests (n = 3).

For initial exposure, three treatment nests were fed 0.8 mL of *Beauveria* sugar-conidia suspension, provided in shallow plastic dishes (3 cm in diameter). Three control nests were provided 0.8 mL of 50:50 sugar water and Triton X-100 solution in the same way. For the week following initial exposure, the temperature and relative humidity of the rearing room were maintained at averages of 23.6 °C (±SE < 0.1) and 46.0 % (±SE 0.6), respectively, consistent with the conditions of the acclimation period. After one week, exposure to the treatment and control solutions was repeated. At the same time, the humidity in the rearing room was increased, and maintained at an average of 66.8 % (±SE 0.9) for one week. We then continued to observe nests for 16 further days during which time relative humidity averaged 81.4 % (±SE 0.5), with average daily temperature of 21.5 °C (±SE 0.1).

The next assay consisted of two nests, one treatment and one control. One of these nests was provided 2 mL of 50:50 sugar water and conidia suspension of the second, unidentified fungus as described above (section 3.3.4). The other nest was provided 2 mL of 50:50 sugar water and pure water as a control. For the following week, daily average humidity was 77.4 % (±SE 0.7) at 21.1 °C (±SE 0.1). The same feeding exposures were then repeated, and nests observed for the following eight days, with 63.5 % (±SE 1.0) average humidity and 21.7 °C (±SE 0.1) mean temperature. All nests were removed from nest boxes and all live and dead adults counted 45 days after beginning the *Beauveria* bioassay and stored at 4 °C.

We compared wasp mortality rates between treatment and control groups for each infection assay using a Kaplan-Meier survival analysis in R 4.1.1 (R Core Team, 2020) with the *survival* and *survminer* packages (Kassambara et al., 2021; Team, 2020; Therneau, 2021). The date of first exposure to conidia suspensions was set as day zero for each assay.

3.4 Results

3.4.1 Fungal identification

We found two species of fungi infecting *P. chinensis* paper wasps collected from Farewell Spit. Through molecular methods we were able to confirm that one of the fungi species belongs to the genus *Beauveria*. We amplified *Beauveria* isolated from two different mycosed wasps. For the first wasp, the resulting ITS sequence was of poor quality with significant noise (thus we did not submit it to GenBank), yet blasted to *Beauveria* sp. A sequence of 529 bp of 18S from this same wasp matched *B. caledonica* with 95.3% identical sites, and 178 bp of *ef1*α matched *B. malawiensis* with 100% similarity. The second *Beauveria* isolate resulted in 506 bp of good quality ITS sequence and matched *B. malawiensis* with 98.2% identical sites. For 18S, a 558 bp long sequence matched *B. caledonica* with 93.3% identical sites, and 178 bp of the *ef1*α gene matched *B. malawiensis* with 100% identical sites. The phylogenetic tree that includes many *Beauveria* species also confirm the identity of our isolate as *B. malawiensis* with a posterior probability of 1 (Fig. 3.3). Considering the phylogenetic tree and the nested PCR protocol for *Beauveria* species identification developed by McKinnon et al. (2018) combined, we can confirm that the *Beauveria* species found parasitising *P. chinensis* is *B. malawiensis*.

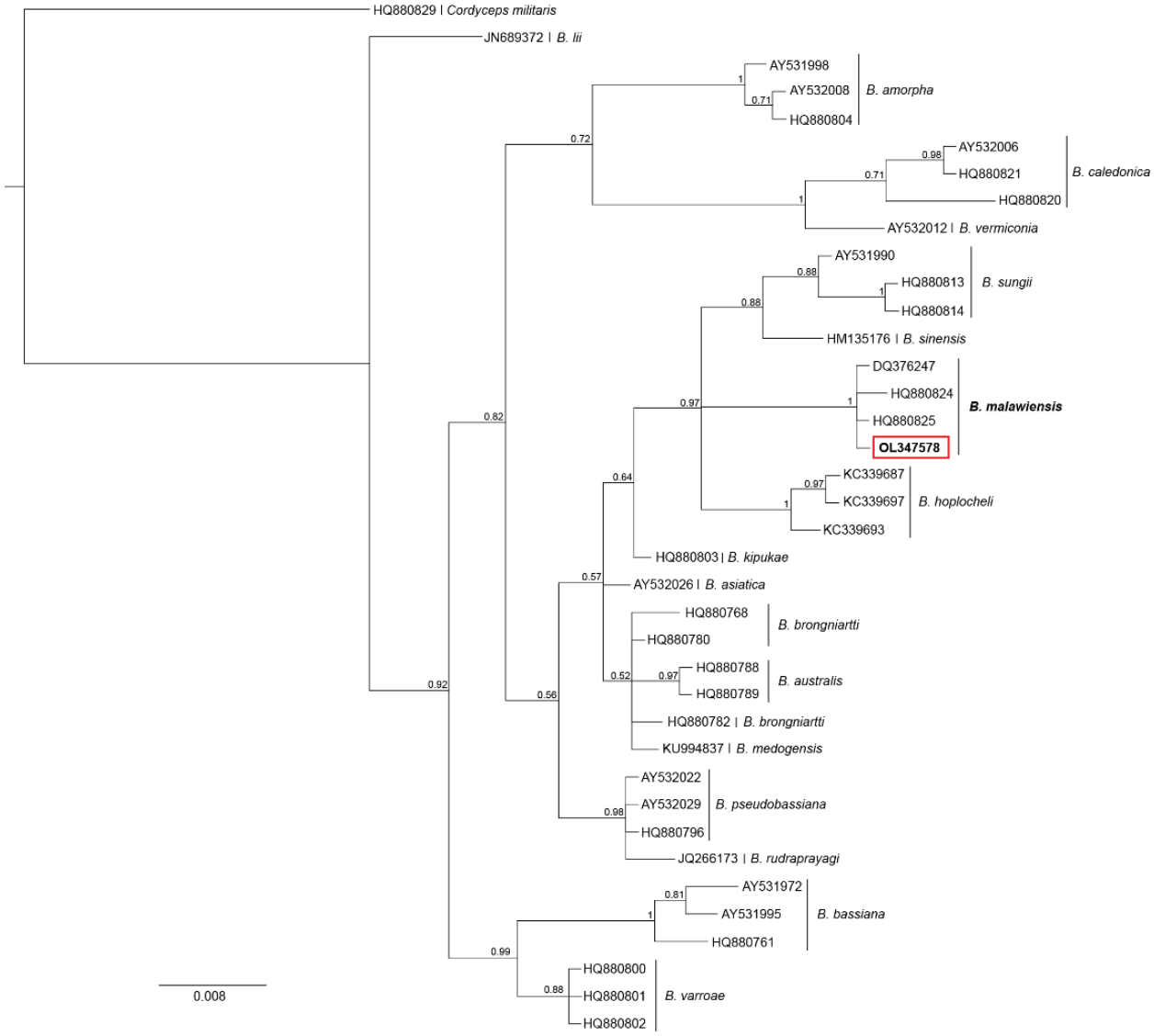


Figure 3.3 Bayesian phylogeny of *Beauveria* species based on the ribosomal DNA internal transcribed spacer (ITS) gene. Each individual isolate is shown by its GenBank accession number, and the species name noted. Posterior probabilities are presented above branches. The *P. chinensis* isolate generated in our study is in bold and inside the red box.

The morphological analysis of samples of *B. malawiensis* confirmed the molecular identification of this fungus, as the characters observed matched those described for the species (Cummings, 2009; Imoulan et al., 2017; Rehner et al., 2006). *Beauveria malawiensis* samples had septate hyphae of 1.2-2.0 µm in width (average 1.6 µm, *n* = 20), occasionally branched. Conidiophores were globose to acutely obpyriform, with denticulate rachides and cylindrical conidia 3.0-3.7 x 1.2-2.1 µm in size (average 3.3 x 1.6 µm, *n* = 20; Fig. 3.4).

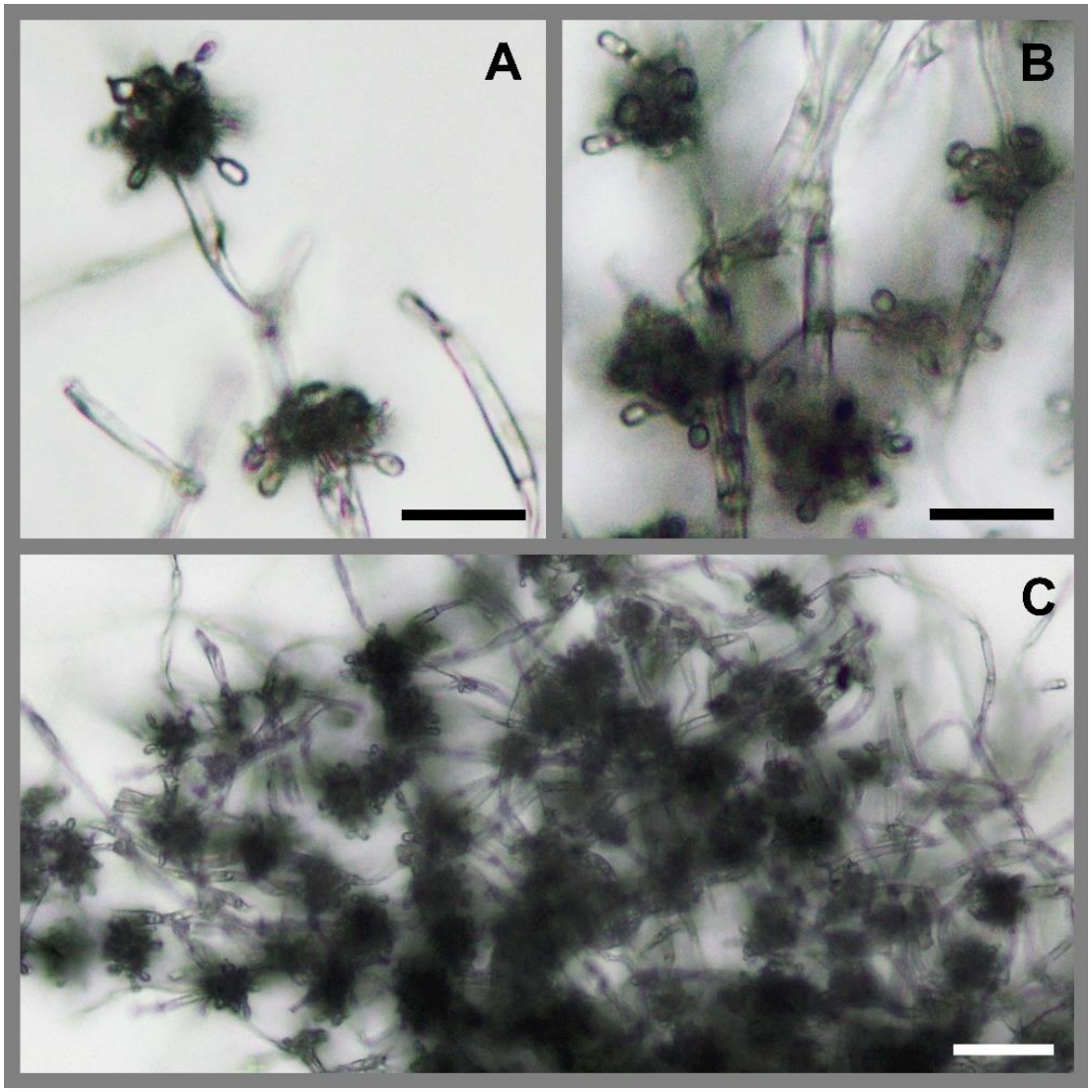


Figure 3.4 Microscope photographs of the fungal body from our wasps, which we identified as *Beauveria malawiensis*. The sample was isolated from a mycosed adult wasp infected in the laboratory and prepared without stain. **A,B)** Globose to acutely obpyriform conidiophores with denticulate rachides and cylindrical conidia 3.3 x 1.6 µm on average. Scale bars = 10 µm; **C)** Septate hyphae with condidogenous cells densely clustered. Scale bar = 20 µm. Photographs: Aiden Reason.

DNA sequence data provided conflicting results for the hirsutelloid fungus. For the first isolate of this species, 266 bp of ITS sequence matched *Hirsutella citriformis* with 96.2% identical sites, and 566 bp of 18S matched the same species with 96.9% identical sites. The second isolate produced an ITS sequence of 394 bp that matched most closely to *Trichothecium crotocinigenum* at 96.6%, and an 18S sequence of 631 bp matched three different species all to 97.4% similarity: *Purpureocillium lilacinum*, *Metarhizium granulomatis*, and *Metarhizium viride*. Of all the conflicting genera matches that resulted from the different genes, only

Ophiocordyceps spp. – revised nomenclature for *Hirsutella* (Quandt et al., 2014; Sung et al., 2007) – provided a logical match to the general morphology and ecology of the fungus in question (see Fig. 3.2). We built a phylogeny for our ITS and 18S isolates, but the alignments were poor, as previously reported for ribosomal DNA data for *Ophiocordyceps* (Li et al., 2013; Sung et al., 2007). The resulting unresolved tree is not presented.

Microscopic examination of isolated synnemata from this fungus confirmed presence of characters matching those described for hirsutelloid *Ophiocordyceps* spp. (Mains, 1951; Meyer et al., 2007; Mollá et al., 2020; Montalva et al., 2017). The mature synnemata of collected specimens were up to 12 mm long and 0.3 – 1.2 mm wide, arising from all over the host body at joints and inter-sclerite membranes, especially from between the thoracic and abdominal tergites. The synnemata were attenuated upwards and usually unbranched, white to brown, seemingly darkening with age. They were formed from longitudinal, densely packed septate hyphae 2.0-3.5 µm (average 2.7 µm, *n* = 20) in width. Conidiogenous cells arose laterally from the hyphae, uniformly dense to occasionally clustered. These ranged from 15 – 40 µm in length, basally inflated and narrowing abruptly to slender necks (Fig. 3.5). Conidia had a mucilaginous coat, were hyaline and allantoid, and measured 3.5-7.1 x 2.2-4.3 µm (average 5.8 x 2.7 µm, *n* = 20). Due to the observed morphology and ecology of this species we putatively identify it as *Ophiocordyceps humbertii* (see discussion section 3.5).

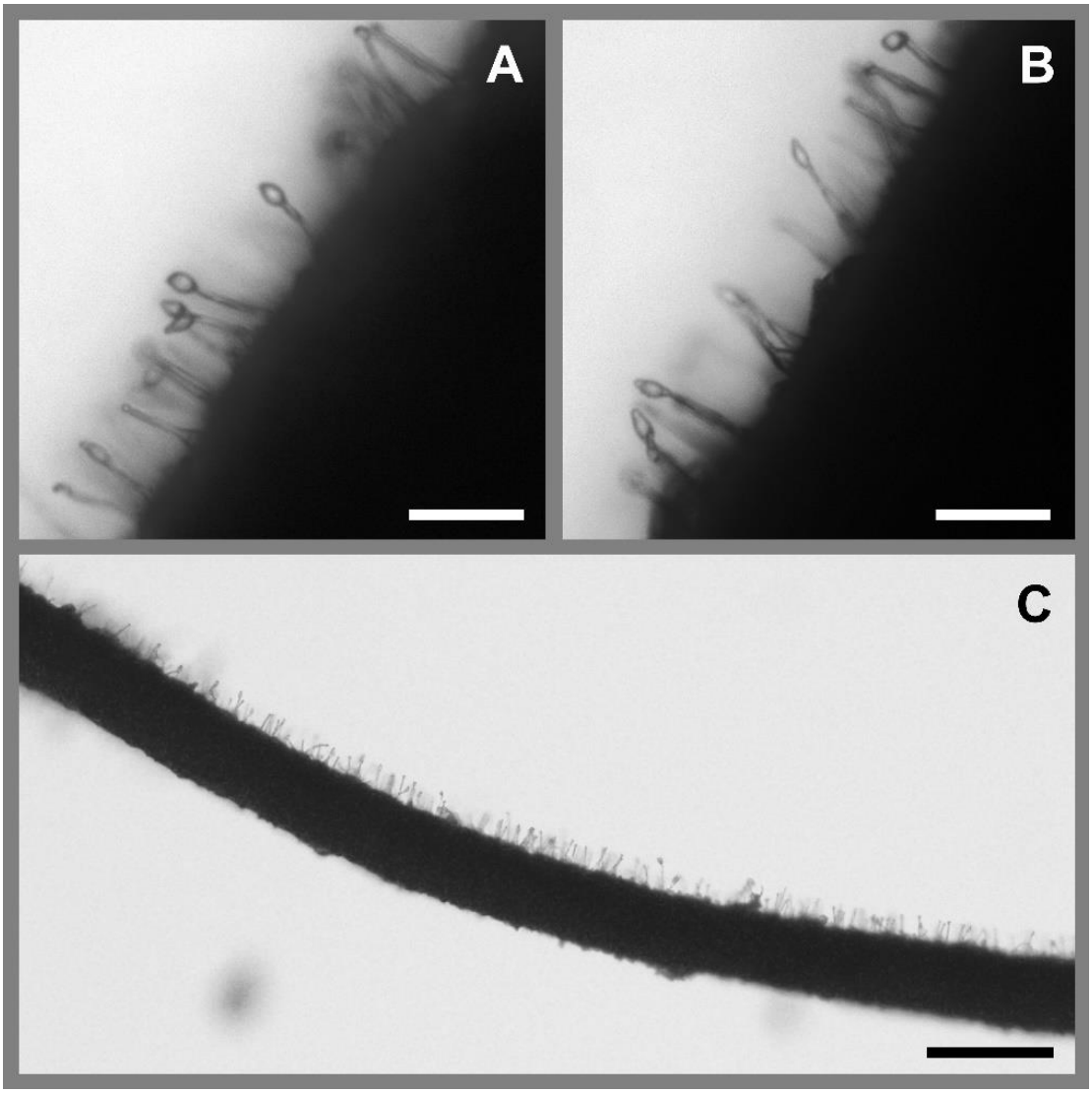


Figure 3.5 Microscope photographs of *Ophiocordyceps humbertii*. The sample was isolated from a mycosed adult wasp collected from the field and prepared without stain. **A,B)** Conidiogenous cells with inflated bases tapering to slender necks, and allantoid conidia with mucilaginous coat 5.8 x 2.7 µm on average. Scale bars = 10 µm. **C)** Conidiogenous cells arising laterally from a synnema, abundant on one side only potentially due to disruption during harvesting. Scale bar = 20 µm. Photographs: Aiden Reason.

3.4.2 Prevalence of fungi in wild nests

During the field study in the austral summer 2020 – 2021, a total of 495 *Polistes chinensis* nests were observed for fungal pathogens on Farewell Spit. Only seventeen nests (and one isolated wasp) were discovered with evidence of parasitisation by entomogenous fungi. Of these infections, three were identifiable as *B. malawiensis* and ten as *O. humbertii* (Table B.1). The remaining five were unidentifiable due to having no external mycelial growth, yet could be recognised as internally mycosed. Diagnostic characteristics for an infection included the

cadavers' compound eyes and ocelli becoming paler brown or silver-coloured, and dullness of the wasp cuticle (Fig. B.1). Early stages of fungi emergence were often observed as the appearance of a fine white tomentum on the hosts' antennae. In the following year, austral summer 2021 – 2022, 17 out of 522 recorded nests had evidence of fungal infection, with four identifiable as *Beauveria* spp., eight as *O. humbertii*, and five unidentifiable.

In the molecular assay, we found no evidence of *B. malawiensis* in the 24 *P. chinensis* larvae from the 12 wild nests examined, nor in the 16 adult wasps from eight wild nests collected from Farewell Spit. The prevalence of *Beauveria* in wild, healthy individuals, detectable using this method and population sample size, was zero.

3.4.3 Infection bioassays

All three treatment nests in the *B. malawiensis* bioassay had an elevated mortality rate of adult wasps compared to controls ($P < 0.001$, Fig. 3.6; Table 3.2). In two of these nests, conidiogenesis was observed from cadavers during the experiment (Table 3.2). Many cadavers from the third treatment nest, which had the highest number of dead adults at the end of the bioassay, later sporulated while in storage. Conidiogenesis was first observed in *B. malawiensis* treatment nests at day 16 after initial exposure. The treatment nest with the lowest mortality rate also had the lowest concentration of cfu per adult wasp due to a slightly larger adult population (Table 3.2). However, this was also the nest with the highest number of sporulating cadavers.

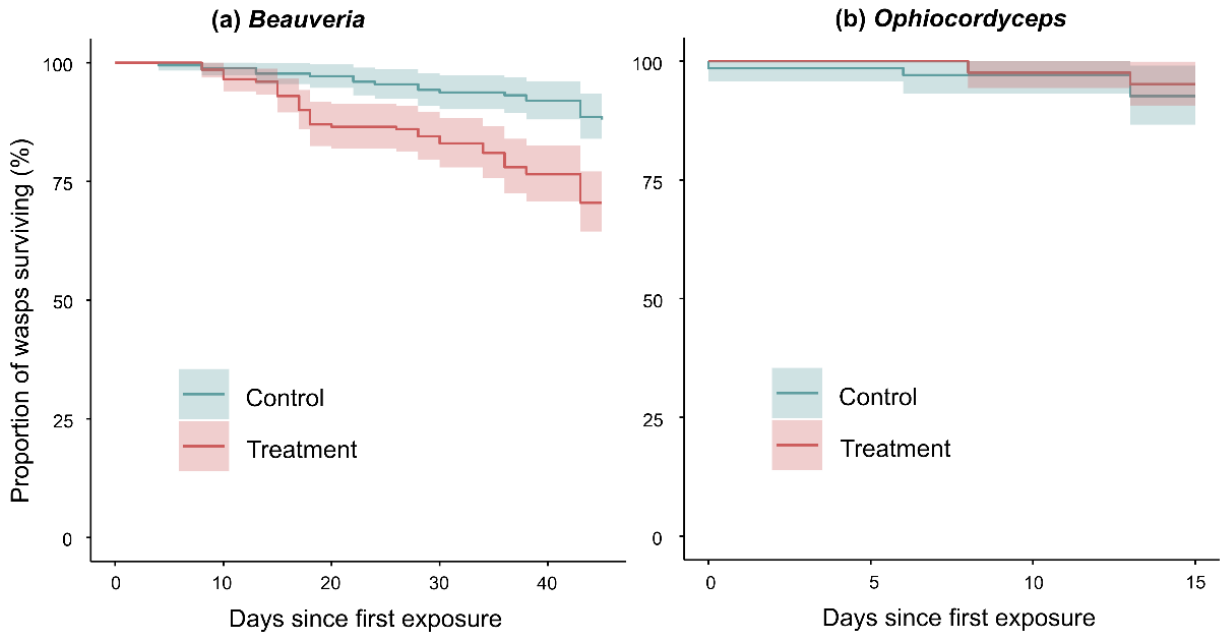


Figure 3.6 Kaplan-Meier survival plots showing the average (± 95% CI) proportions of individual adult wasps surviving over time in each treatment group for each infection bioassay. **A)** *Beauveria* assay; *n* = 3 nests per group. Survival probability begins to drop for treatment nests after the second exposure at day seven, when relative humidity in the rearing room was consistently above 65% and as high as >90%. Log-rank significance test for difference between groups: $P < 0.001$. **B)** *Ophiocordyceps* assay; *n* = 1 nest per group. There was no difference in wasp survival rates between treatment and control nests, and no indication of positive infection observed (log-rank significance test: $P < 0.511$).

Table 3.2 Data for proportion of adult wasp deaths per nest over the course of each assay and concentrations of cfu (conidia forming units) per adult wasp, for nests in treatment groups. *n* = number of adult wasps per nest. P_{dead} = the number of dead wasps as a proportion of the total. Days is the number of days since first exposure to conidia treatment. Conidiogenesis is defined as the visible emergence of mycelia from a dead, infected host.

Fungal species	Nest ID	n_{total}	n_{dead}	P_{dead}	Days	cfu wasp^{-1}	Conidiogenesis
Beauveria malawiensis	167	62	34	0.55	45	6.92 x10^4	No
	193	81	10	0.12	45	5.29 x10^4	Yes
	277	57	57	0.26	45	7.52 x10^4	Yes
Ophiocordyceps humbertii	293	83	4	0.05	15	7.33 x10^4	No

There was no significant effect of treatment in the *Ophiocordyceps* bioassay ($P = 0.511$, Fig. 3.6). Both nests had the same mortality rate and no conidiogenesis of cadavers was observed.

In the two nest boxes containing nests with pre-infected foundress cadavers kept for observation only, there was no conidiogenesis observed, and no apparent evidence of infection of live adults. All nests in these boxes had adult mortality rates similar to control nests in applied bioassays.

3.5 Discussion

We identified two species of entomogenous fungi found infecting *Polistes chinensis* on Farewell Spit, New Zealand. The first species was identified through molecular methods as *Beauveria malawiensis*. This identification was confirmed by examination of key microscopic characters, especially the size and shape of conidia (Cummings, 2009; Imoulan et al., 2017). The known host range of *B. malawiensis* in New Zealand includes members of Coleoptera, Hemiptera, Hymenoptera, Orthoptera and Phasmatodea, with *Vespula* wasp species recorded as particularly frequent hosts (Cummings, 2009; McKinnon et al., 2018).

Molecular analysis of the second fungal species yielded inconclusive results but provided a point of reference for further exploration. Molecular identification is inherently limited by the availability and quality of sequence data in reference databases (Meiklejohn et al., 2019; Nilsson et al., 2006; Ryberg et al., 2009). This hurdle may be especially complicated for species with complex taxonomic nomenclature (Hibbett & Glotzer, 2011). Following phylogenetic analysis and unification of dual nomenclature, *Hirsutella* species have been revised as hirsutelloid asexual morphs of *Ophiocordyceps* (Araújo & Hughes, 2017; Quandt et al., 2014). The distinctive morphology, microscopic characters, and ecology of this species strongly suggests the identity *Ophiocordyceps humbertii*, in the hirsutelloid form previously known as *H. saussurei,* the only *Hirsutella* species recorded from wasp hosts (Hodge, 1998; Mains, 1951; Montalva et al., 2017; Rose et al., 1999; Speare, 1920; Sung et al., 2007). Similarly, *Ophiocordyceps humbertii* is the only hirsutelloid *Ophiocordyceps* known to infect Vespid wasps (Araújo & Hughes, 2019; Montalva et al., 2017). It was first described by Speare (1920) as a parasite of *Polistes* spp., and is frequently reported infecting Polistine and Vespine wasps (Mains, 1951; Mollá et al., 2020; Montalva et al., 2017; Rose et al., 1999; Sobczak & Somavilla, 2020; Somavilla et al., 2020a). This fungi is recorded as an exotic species in New Zealand (NZOR, 2012b; Pennycook & Galloway, 2004), previously known solely from Vespine hosts (Rose et al., 1999). To our knowledge, this is the first record of *O. humbertii* infecting *Polistes* wasps in New Zealand.

The identification of *O. humbertii* can be further supported from observations by Somavilla et al. (2020a). These authors noted pre-mortem behaviour manipulation of *Polistes* hosts by *O. humbertii* in Brazil. In species with nests initiated by a sole foundress (such as *P. chinensis*), mycosed individuals were observed having affixed themselves to nest combs, in both founding and superindividual phase colonies, as we observed in this study. Only the asexual hirsutelloid form of the fungus was observed in these cases. The mucilaginous coat on *O. humbertii* conidia enables adherence to a new host upon contact (Boucias & Pendland, 1991; Khachatourians & Qazi, 2008; Simmons et al., 2015). Thus, manipulating host behaviour to induce conidiogenesis directly on a hosts' nest likely increases the chances of contact with a new host. Somavilla et al. (2020a) hypothesised that these specimens may represent a cryptic lineage within *O. humbertii*, adapted for improved propagule dispersal in sole-founding social wasp species. During the field study we discovered two specimen wasp nests wherein this proposed strategy may have been successful, with infected cadavers of both the foundress

wasp and an emerged adult present on the nest comb (Fig. B.2). Interestingly, we have also observed infected individuals of *P. dominula* in the Nelson region of New Zealand, exhibiting different pre-mortem behaviour consistent with that described for solitary wasps, and swarm-founding Polistine wasp species (Sobczak & Somavilla, 2020; Somavilla et al., 2020a; Somavilla et al., 2020b) (Fig. B.3).

The prevalence of the identified fungal species in the Farewell Spit paper wasp population is, from present evidence, very low. Nests with evidence of fungal infection constituted only about 3.3% of the total nests recorded in each year of the field study, with specimens identifiable as *B. malawiensis* and *O. humbertii* contributing 0.7% and 1.8% on average, respectively. Our attempt to experimentally detect potential asymptomatic carriers of *Beauveria* spp. in a sample of adult and immature individuals (n = 40) from this population yielded negative results. Given that this fungus was identified in only 0.7% of nests discovered in the field, it is likely that our sample size was too small for positive detection with the molecular method used. There may have also been other limitations to this assay, such as a negligible proportion of hosts exposed to *Beauveria* spp. remaining asymptomatic, or the time of sample collection being unideal for fungus propagation.

In the *B. malawiensis* infection assay, all three treatment nests exposed to the *B. malawiensis* conidia solution experienced significantly higher mortality rates than control nests. While there was no significant main effect of relative humidity on wasp mortality, a substantial increase in mortality rates for treatment nests beginning at approximately day 13 (Fig. 3.6) coincided with an also substantial increase in humidity at the same time. This humidity spike was the result of a period of extreme wet weather locally. Temperatures between 20 – 30°C and high relative humidity are generally required for the germination, hyphal growth, and sporulation of most fungi, including entomopathogens (Goettel et al., 2005; Vega et al., 2012). Exact thresholds vary, but some species may require relative humidity of up to 90% or more (Goettel et al., 2005; Inglis et al., 2012; Vega et al., 2012).

The concentration of *B. malawiensis* conidia in the sugar solution fed to treatment wasps was 2.68 x 10^6 cfu mL^{-1}. A set volume of 1.6 mL solution divided over two feedings per nest resulted in concentrations varying between treatment nests from 5.29 x 10^4 to 7.52 x 10^4 conidia per wasp. These concentrations fall within already determined successful ranges for pathogenicity in similar bioassays, of over 9.05 x 10^3 cfu $wasp^{-1}$ for *B. bassiana* and *B. malawiensis* in *Vespula* hosts (Cummings, 2009; Harris et al., 2000; Rose et al., 1999), to 4 x10^7

cfu wasp^{-1} for *B. bassiana* in *P. dominula* larvae (Mhlongwe, 2018). All the concentrations used for treatment nests in this study resulted in successful fungi infections, and some mycosed cadavers from each treatment nest sporulated. The resulting external fungal bodies were a morphological match to original samples. We consider that this experimental result satisfactorily confirms disease causation by the *B. malawiensis* samples collected from *Polistes chinensis* hosts on Farewell Spit. Previous infection assay studies have satisfied Koch's postulates for microbial pathogenicity of *Beauveria* species to wasps in laboratory settings (Cummings, 2009; Harris et al., 2000; Mhlongwe, 2018; Rose et al., 1999). The present study differs in the husbandry of wasp colonies, which aimed to mimic field conditions as much as practical. Adults were kept with their natal nests and intact combs in relatively large (9 L) containers, with protein and carbohydrate food and nest-building resources provided. Thus, disease causality was confirmed in conditions closer to realistic colony dynamics than previously attempted in known studies.

In our *O. humbertii* bioassay there was no significant effect of fungi treatment on wasp mortality, and no evidence of fungal infection was observed. There may have been many different factors that contributed to this result. However, we hypothesise that a key factor is that conidia of *O. humbertii* are unable to germinate and infect the host via the oral tract. While in other studies it has been shown that some *Beauveria* species can successfully colonise hosts inoculated via this delivery method, this ability is unusual for entomogenous fungi in Hypocreales (Harris et al., 2000; Mannino et al., 2019; Mora et al., 2018). As discussed in section 3.5, it is more likely that *O. humbertii* conidia are dispersed by transfer from a mycosed wasp to a new host via direct contact (Boucias & Pendland, 1991; Khachatourians & Qazi, 2008; Simmons et al., 2015; Somavilla et al., 2020a). In contrast, *Beauveria* colonisation of paper wasps in the field is more likely a result of contact with propagules away from the nest, on vegetation or via foraged prey (Harris et al., 2000; Mayorga-Ch et al., 2021).

Polistes chinensis is an invasive species in New Zealand. There is no effective landscape-scale control for this species currently available (Beggs et al., 2011; Toft & Harris, 2004), although management options are under ongoing investigation (Toft, 2020). Biological control is considered the most viable avenue for development to manage paper wasp populations (Brown, 2021). The open-air nesting behaviour of *Polistes* makes these wasps more susceptible to predation or parasitism by other organisms, in comparison to ground-nesting wasps such as

Vespula spp., for which previously introduced biocontrol agents have had non-significant population impacts (Beggs et al., 2011; Brown, 2021).

As biological control agents, fungal entomopathogens are typically applied as inundative measures in a similar manner to a chemical pesticide (Inglis et al., 2001; Jaronski, 2010; Lacey et al., 2001). This method contrasts with classical or inoculative biocontrol applications wherein there is an expectation for epizootic spread (Behle & Birthisel, 2014; Goettel et al., 2005; Hajek, 2004; Jaronski, 2010; Rose et al., 1999). For inundative release, mass production of the chosen fungi is required, and there are several major constraints on this process. Although fungi of Hypocreales are usually more easily cultured than other entomogenous groups (such as Entomophthorales), the cultivation of many species is still difficult (Goettel et al., 2005; Hajek, 2004; Pell et al., 2001). The formulation of a carrier material, solution, or other means of delivery must be properly suited to the infection pathways of the fungal agent. The delivery method must consider long-term propagule viability, relatively narrow environmental windows for completion of the fungi life cycle, and the level of host specificity (Brancini et al., 2021; Goettel et al., 2005; Inglis et al., 2001; Jaronski, 2010, 2014). While generalist entomopathogens such as most *Beauveria* species (Rehner & Buckley, 2005; Rehner et al., 2011; Vega et al., 2012) are often used as biological control agents in agricultural or horticultural settings, they may not be suitable as biocontrol agents for invasive species in conservation contexts due to the potential for non-target effects. Species with narrow host ranges should be prioritised for further research and testing to minimise risks to non-target arthropod fauna (Hajek et al., 2021; McEvoy, 1996; Pearson & Callaway, 2003), though there may be substantial diversity in different strains of the same fungal species (Bidochka et al., 2002; Maurer et al., 1997). Recent research suggests that some entomogenous fungi may prove viable candidates for further development as biocontrol agents of invasive *Polistes* wasps (Mhlongwe, 2018). There is an interesting potential for further work in this area, particularly for relatively understudied species such as those identified in this study.

Chapter 4: General discussion

In this thesis my aim was to quantify the presence of invasive *Polistes chinensis* paper wasps in the Onetahua (Farewell Spit) Nature Reserve, identify factors influencing their survival, and assess the level of potential ecological impact these wasps are having on local invertebrate communities.

In Chapter two I investigated the nesting ecology and invertebrate prey community of *P. chinensis* within the study site. I found an average colony survival rate of approximately 20%, similar to nest survival rates found in previous studies in Aotearoa. Phase shifts in colony development indicated that the degree of predation pressure exerted by *P. chinensis* colonies was likely to be greatest in the month of February each year, when foraging wasps are most abundant. I estimated that around this time, the density of active colonies was approximately 23 nests ha^{-1} in the study area.

Metabarcoding analysis of wasp larvae gut samples collected in December, January, and February 2020 – 2021 revealed that the most ubiquitous taxa in the *P. chinensis* prey community on Onetahua were endemic *Rhodopsalta* cicada species, several native and exotic moth species, and copper butterflies (*Lycaena* spp.). Cicadas have only been previously recorded in the Aotearoa paper wasp prey community in Nelson (Howse et al., 2022). There was no direct evidence obtained from this analysis that this prey community includes species of conservation importance. However, 60% of all unique taxa sequences in gut samples could not be identified from the current sequence reference database. I hypothesise that some of these taxa are likely to be rare and understudied species due to the absence of corresponding barcode sequences in the GenBank reference library. Barcoding approaches thus may not give a complete picture of the conservation impacts of this invasive wasp. The high prevalence and abundance of wasps around February each year might indicate that rare species that occur at this time could be at risk.

In the final chapter I identified the two species of entomopathogenic fungi that I observed to be fatally infecting paper wasps on Onetahua. Using both molecular and morphological approaches, my analysis indicated that these species were *Beauveria malawiensis* and *Ophiocordyceps humbertii*. Colony survey and monitoring data showed that these fungi infected individuals in 3.3% of all colonies found. In bioassays in which healthy, whole paper wasp colonies were passively exposed to propagules of these fungal species via a

food source, successful infection with *B. malawiensis* was achieved in all treatment colonies, with wasp mortality rates of up to 55%.

4.1 Population dynamics of Polistes chinensis in the Onetahua Nature Reserve

My estimates of colony abundance, survival, and productivity observed for *P. chinensis* were similar to overall average colony metrics previously recorded in Auckland and Nelson regions (Clapperton, 1999; Clapperton & Dymock, 1997; McGruddy et al., 2021a). Average nest sizes on Onetahua were slightly larger than those found in Nelson, likely due to competition from the high abundance of *P. dominula* in the Nelson region (McGruddy et al., 2021a), which I did not observe to be present in the Onetahua reserve. Average nest sizes were smaller and the survival rates slightly lower than those found in Northland colonies (Clapperton & Dymock, 1997; Clapperton & Lo, 2000), which may be due to the comparatively cooler and wetter climate of Onetahua (Mackintosh, 2001). A range of biotic and abiotic factors will likely contribute to variation in densities between sites.

While colony survival rates were not significantly different between years in this study, the slightly slower development rate of colonies in the first year may reflect interannual differences in weather conditions (NIWA, 2022), particularly in the most critical period for colony survival in late spring and early summer. Spring weather conditions have previously been highlighted as a major predictor for yearly variation in populations of *Vespula vulgaris* wasps (Lester et al., 2017). The dependence and responsiveness of *P. chinensis* to climatic conditions observed elsewhere (Hozumi et al., 2015; Jeanne & Morgan, 1992) suggests that average colony survival and productivity may change significantly in response to climate, as has been predicted for *Vespula* wasps (Lester & Beggs, 2019). Weather conditions such as rainfall and humidity levels may impact fungal pathogen transmission in this and other study systems. These impacts may occur directly, due to the environmental thresholds for fungal development, or indirectly, through colony survival and density.

Approximately 3% of all nests observed in each year had at least one individual that showed evidence of fungal infection. These individuals were primarily discovered in November and December each year when average humidity was higher than later in summer. Conidiogenesis of infected wasps on monitored nests was noted to occur predominantly after periods of rain. It should be noted that the number of wasps that succumbed to infection and died away from the nest could not be measured, as only one such cadaver (mycosed by *B.*

malawiensis) was ever observed. It is also possible that nests with infected individuals may have had a lower likelihood of discovery in the field, as nests in the shade of vegetation were potentially more likely to succumb to infection than those in direct sunlight. Fungal conidia are sensitive to ultraviolet (UV-A and -B) light and such exposure can reduce conidia viability in many species (Goettel et al., 2005; Hajek, 2004). Thus, the actual prevalence may be higher than recorded in this study. High nest densities and worker drift between colonies with overlapping foraging ranges can lead to increased pathogen transmission and connection of microbial networks, reducing colony fitness, as found in *Vespula vulgaris* wasps in their introduced range (Gruber et al., 2019). Several studies have found high rates of intercolonial drift in *Polistes* wasps (Kasuya, 1981; Sumner & Cini, 2021; Sumner et al., 2007).

The enemy release hypothesis refers to the process in which a species goes through a bottleneck event when arriving to new environments, thereby leaving behind many natural enemies from their native range (Keane & Crawley, 2002; Torchin et al., 2003). However, evidence for enemy release was not found for invasive *V. vulgaris* wasps in their invaded range, despite the exceptionally high population reached in Aotearoa (Lester et al., 2014). The overall lower colony survival rates of *Polistes chinensis* in Aotearoa compared to their native range (Clapperton & Dymock, 1997; Miyano, 1980) may suggest a similar lack of enemy release, or the acquisition of new pathogen associations. Lower genetic diversity, also typically induced by bottleneck introductions, may contribute to possibly higher pathogen loads (Dobelmann et al., 2017). Due to only two introduction events of *P. chinensis* to Aotearoa, the genetic diversity of the country-wide population is low (Tsuchida et al., 2014).

The hypothesised main dispersal pathway of asexual *O. humbertii* involves contact of a new potential host with the fungi synnemata, from which the sticky mucous-coated conidia may adhere to the new host. Thus, infection rates of this species in the wild may positively correlate with higher wasp densities. However, density-dependent disease resistance is a well-recorded phenomenon among gregarious and social insects, whereby hygienic behaviours and other disease resistance factors increase in a population reaching high densities (Pie et al., 2005; Wilson et al., 2002). Hence, the potential for these fungi to have a regulatory effect on wasp populations in natural settings is unlikely (Stiling, 1988). Wasp contact with *Beauveria* conidia is more likely to be from soil or vegetation while foraging, or from infected prey species (Mayorga-Ch et al., 2021; Pell et al., 2010). These courses are more independent of nest density

and the presence of other wasp colonies within typical *P. chinensis* foraging distances. Rates of *Beauveria* infection may thus be more stable across varying wasp population densities.

Colony hygiene behaviours are likely to have a significant impact on rates of disease in *Polistes* by inhibiting pathogen infection and transmission (Manfredini et al., 2013; Mayorga-Ch et al., 2021). Hygienic behaviours of wasps and other social insects responding to the presence of microbial pathogens can include personal and communal grooming, expulsion of infected nestmates, and corpse removal (Cremer et al., 2007; Mayorga-Ch et al., 2021; Mayorga-Ch et al., 2020; Renucci et al., 2011). In my *B. malawiensis* infection assay, wasps in treatment colonies were frequently observed dragging the cadavers of dead nestmates around the nest box floor, possibly in an effort to remove them from the nest vicinity (Manfredini et al., 2013; Mayorga-Ch et al., 2020; Renucci et al., 2011). No dead cadavers were observed clinging to the nest comb in the manner observed in wild-infected specimens. The absence of such cadavers in bioassays may be the result of infected individuals being excluded from the nest by other wasps. It should be noted that these were "caged" experiments that may not have entirely reflected natural scenarios and processes (Jandt et al., 2015).

4.2 Conservation impacts of Polistes chinensis and threat to native invertebrates

As the difference in wasp predation pressure between colony phases is likely substantial, the timing of milestones in colony development may have significant implications for efforts to mitigate predation impacts on native invertebrates. For example, a study from *P. chinensis'* native range in Japan estimated that the amount of prey consumed by a colony during the founding phase constitutes only about 5% of the total prey weight consumed over the whole season (Suzuki, 1984). Consequently, the conservation threat posed by these wasps may be negligible early in the season. The amount of prey gathered has also been found to drop substantially after the emergence of males and gynes as foraging priorities are switched to carbohydrate resources (Clapperton, 1999; Hoshikawa, 1981).

Shifts in species' phenology due to climate change have been observed in many insects around the world, including for Hymenoptera species (Bartomeus et al., 2011; Stemkovski et al., 2020; Tryjanowski et al., 2010). Increases in average spring and summer temperatures may advance colony initiation or speed colony development (Tryjanowski et al., 2010), potentially shifting the period of greatest predation pressure exerted by colonies. In my study system, such shifts may mean that the proportional composition of *P. chinensis'* prey community in Aotearoa

changes. Likewise, potential phenological shifts in prey species could alter the temporal composition of the prey community, even if wasp phenology does not substantially change.

A strong temporal trend also emerged in the molecular analysis of the wasp diet, particularly in relative read abundance across sampling months. This trend and the intersections of prey community composition per month displayed in the Venn diagrams (Fig. 2.11) showed that there are substantial temporal differences in the *P. chinensis* prey community over the course of the nesting season. Decision makers on potential future management of paper wasp populations should consider the timing of control methods or applications to maximise method efficiency and mitigate the impact of predation on native invertebrates. For example, enacting control methods in the spring may spread efforts over a larger number of nests that will naturally decrease by 50% by early summer anyway; yet controls applied later in the season when the surviving colonies are more robust may fail to alleviate impacts of colony predation. Application of controls towards the end of the founding or beginning of superindividual periods for most colonies, approximately at the end of December or early January, may strike a balance between these possibilities. However, the ideal timing for potential management interventions will also depend on the control method or agent being employed.

Several sources have anecdotally reported that *Lycaena* spp. abundance have been significantly impacted by *P. chinensis* predation (Arter-Williamson, 2016; Early, 2007; Parkinson & Horne, 2007). While not yet proven at the population level, the presence of *Lycaena* spp. in all *P. chinensis* diet analyses conducted thus far does support the likelihood of substantial predation pressure by *P. chinensis* on these butterflies (Howse et al., 2022; Lefort et al., 2020; Ward & Ramón-Laca, 2013). The impact of predation on *Lycaena* spp. may be particularly strong due to the high abundance of wasp nests founded in *Muehlenbeckia* spp. plants, as observed in this and other studies (Howse et al., 2022; McGruddy et al., 2021a). *Muehlenbeckia* are the larval food plants of *Lycaena* spp., and adults typically do not stray far from their natal plant (Gillespie & Wratten, 2012; Patrick & Patrick, 2012). *Muehlenbeckia complexa* is one of the most abundant plants on Onetahua, with some *M. australis* also present in patches at the base of the spit.

Paper wasp predation has been shown to have significant population-level ecological impacts in Aotearoa. Populations of common blue butterflies (*Zizina labradus*) in the Nelson region are estimated to have plummeted by 87% since the arrival of *P. dominula* (McGruddy et

al., 2021b). *Zizina* butterflies were not among the species identified in the diet analysis in my study site. However, the larval food plants of common blue butterflies include clovers (*Trifolium* spp.), tree lucerne (*Cytisus proliferus*), and bird's-foot trefoils (*Lotus* spp.) (Arter-Williamson, 2011; Gillespie & Wratten, 2012), the latter of which was frequently observed as a focus of *P. chinensis* foraging during data collection in this study. *Polistes dominula* was not observed on Onetahua during data collection. However, if this species becomes established in the reserve, the overlap in the diet of these wasps (Howse et al., 2022) will likely substantially increase the predation pressure exerted on the prey community.

The high proportion of mOTUs found in the diet analysis with no suitable DNA barcode match in GenBank suggests that there are a high number of species present on Onetahua that have not had genetic sequences submitted to this database, and may be undescribed. Further work to unify taxonomy with molecular sequencing for DNA barcode reference libraries would substantially improve the useability of metabarcoding analyses for species-specific requirements (Kvist, 2013; Kwong et al., 2012). Other studies in Aotearoa have highlighted the lack of barcode sequences for many insect taxa in reference libraries (Dopheide et al., 2019; Wöger et al., 2020). Future updates to these libraries could enable re-analysis of the diet samples from this study with a higher proportion of mOTUs matching sequences identified to species, as evidenced by my re-analysis of diet data from Howse et al. (2022) identifying sequences of *Rhodopsalta* species (Bator et al., 2021). There was only a year between analyses, but my re-analysis using updated databases found many more species.

Non-alpine shrub and grassland and coastal scrub habitats contain 60% of native Lepidopterans designated as 'At Risk' (Patrick & Dugdale, 2000), including congenerics of several taxa identified in the diet analysis (Hoare et al., 2017). Given the coastal habitat and unique assemblage of flora and fauna at Onetahua (Brown, 1978; Kelly, 1991), it is not surprising that rare insect species have already been identified from this area (Patrick & Patrick, 2019). The possibility that paper wasps are attacking and eating such species, potentially with population-level impacts, cannot be ruled out.

I do not believe that the results of my study currently provide sufficient evidence that the *P. chinensis* population on Onetahua presents a clear or immediate and serious threat to species of conservation importance in the native invertebrate community. It may be more prudent at this time for conservation decision-makers to focus funding and efforts into the removal of other invasive species, such as rats, stoats, and feral pigs (*Sus scrofa*), from the

Nature Reserve and surrounding area. During data collection, myself and others observed substantial ground disturbance caused by the rooting of pigs across many areas of the spit (Fig. 4.1). Feral pigs have been found to severely affect invertebrates through predation and compaction and disruption of soils, as well as their impact on birds through egg predation and disturbance of nesting sites, and extensive impacts on plant species and communities (Wehr et al., 2018). It is likely that the pigs are eating larvae of dune scarab beetles (*Pericoptus* spp.) in this area. It has been noted that feral pigs are impacting carabid beetle larvae *Megadromus speciosus* through predation in the Marlborough sounds (N.P.C.A., 2018), and potentially larvae of the stag beetle *Dorcus helmsii* in lowland forests (Parkes et al., 2015).

While my study found no compelling evidence for a clear, immediate, or serious conservation threat posed by *Polistes chinensis*, I would note that there may well be some native invertebrates strongly affected, but which are not represented in current databases.

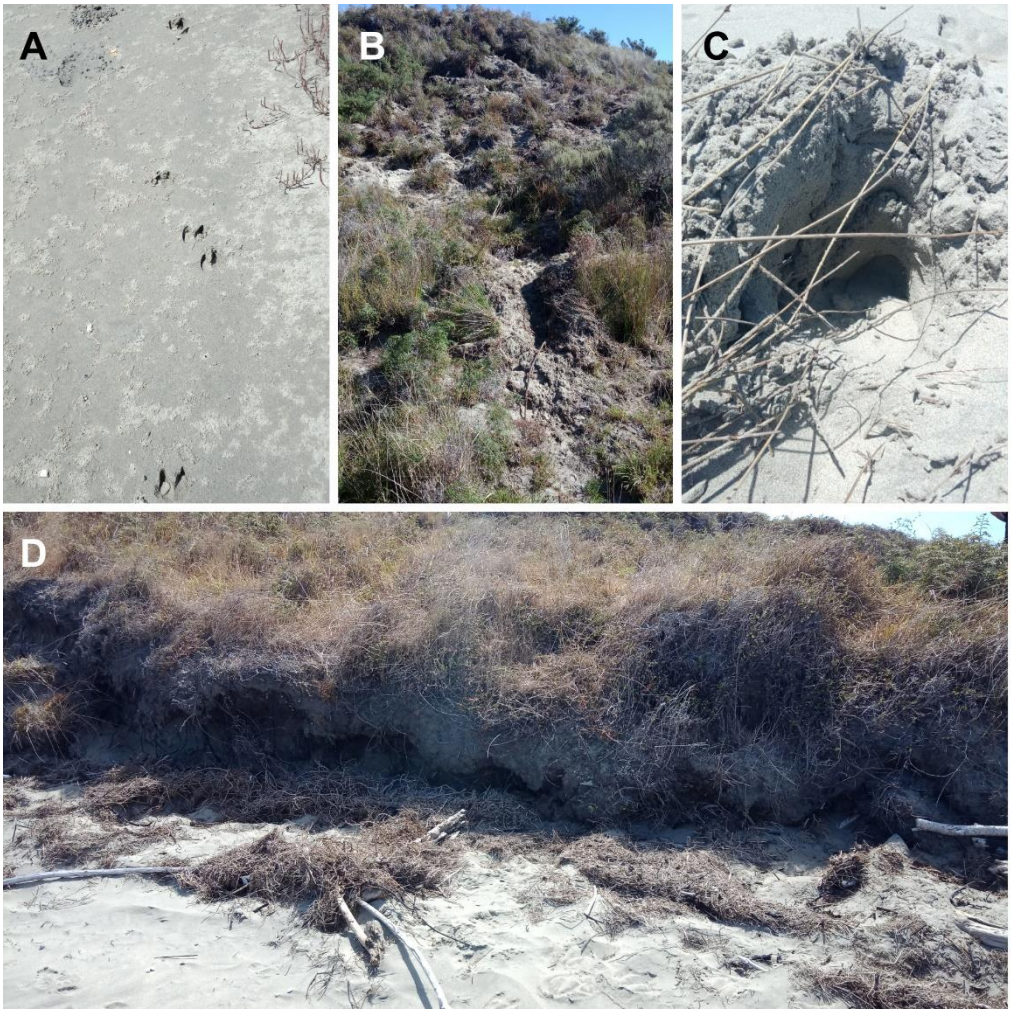


Figure 4.1 Photographs documenting feral pig (*Sus scrofa*) sign and ground disturbance on Onetahua, January and February 2022. **A)** Pig tracks, approximately 4-5 cm wide from toe to toe; **B)** Ground

disturbance from rooting over a sloped area of approximately 50 m^2; **C)** Dig site with impression in the shape of a pig snout; **D)** Deep ground disturbance across the edge of vegetation on the spit's southern beach, for a distance of over 20 m (approximately 4-5 m pictured). Photographs: Aiden Reason.

4.3 Potential for entomopathogenic fungi as biological control agents for invasive Polistes wasps

Ultimately, as stated above (section 4.2), I do not believe that my results provide sufficient evidence to justify any immediate high-intensity efforts to control *P. chinensis* wasps within the Onetahua Nature Reserve. I believe that greater value could be achieved by directing conservation resources to other areas and supporting existing projects for the time being. Nevertheless, the rapid range expansion and significant impacts of *Polistes dominula* are likely to amplify the ecological impacts of *P. chinensis* in their sympatric ranges (Howse et al., 2022). Thus, longer-term investment in research and development of methods to control populations and impacts of these species, particularly in areas of higher colony densities, remains a worthy endeavour.

Biological control by natural enemies is currently considered the most viable avenue of research for control agents for *Polistes* wasps in Aotearoa (Brown, 2021). Other studies have found support for the potential of entomogenous fungi as biological control agents for *Polistes* species (Mhlongwe, 2018), and also presented evidence for non-target effects of fungal biocontrols on these wasps (Mayorga-Ch et al., 2021). Mhlongwe (2018) found that key elements likely to be limiting the effectiveness of entomopathogenic fungal biocontrols in the field are abiotic (temperature and humidity) conditions required by the fungi for germination, and biotic factors such as the confounding presence of other microorganisms in wasp nests. Many entomopathogenic fungi require relative humidity of 95% or more to germinate and complete the life cycle (Goettel et al., 2005; Inglis et al., 2012; Jaronski, 2010). The high humidity average of 80% and above on Onetahua (NIWA, 2022) may have contributed to the levels of fungal infections observed.

A factor of high importance in biological control selection is host specificity (Hajek et al., 2021). Broad-target mycoinsecticides are frequently used in commercial biocontrol applications for agricultural pests due to the wider range of target insects (Uma Devi et al., 2008; Vega et al., 2012). However, in a conservation context, biological controls should be as host-specific as possible to limit non-target effects and avoid further ecological disruption (Howarth, 1991;

Louda et al., 2003). In the study case, thorough testing of the virulence and pathogenicity of any species or strain of entomopathogenic fungi to non-target Hymenopterans and other invertebrates would be necessary (Brown, 2021). For example, non-Vespid Hymenopterans observed during data collection in this study included the native wasps *Tachysphex nigerrimus* (black cockroach-hunter wasp) and *Pison spinolae* (Crabronidae), *Epipompilus insularis* (Pompilidae), *Degithina sollicitoria* (Ichneumonidae), *Pseudofoenus* sp. (Gasteruptiidae), as well as native bees *Leioproctus* spp. and *Hylaeus relegatus* (Colletidae). *Ohiocordyceps humbertii* has a relatively narrow host range, being recorded primarily from hosts in the family Vespidae, though hosts from Ichneumonidae, Pompilidae, and Crabronidae have also been reported (Humber, 2005; Montalva et al., 2017; Sobczak & Somavilla, 2020; Somavilla et al., 2020a; Somavilla et al., 2020b). As there are no social wasp species native to Aotearoa, this host range may be a desirable trait for a biocontrol for invasive *Polistes* and/or *Vespula* wasps. *Beauveria* species typically have much broader host ranges, spanning multiple insects and Arthropod orders (Mascarin & Jaronski, 2016; Rehner et al., 2011). *Beauveria malawiensis* has been recorded from several host orders in Aotearoa (Cummings, 2009). However, some *Beauveria* species, strains, or isolates are much more host-specific or adapted to specific environmental conditions (Bidochka et al., 2002; Imoulan et al., 2017).

While both fungal species identified in this study have been previously recorded in Aotearoa from *Vespula* hosts (Cummings, 2009; Glare et al., 1993; Rose et al., 1999), to my knowledge there was previously no formal record of either *B. malawiensis* or *O. humbertii* infecting *Polistes* hosts in Aotearoa, though both were known to infect *Vespula* spp. The New Zealand Organism Register lists both of these fungi as of exotic origin in this country (NZOR, 2012a, 2012b), although there is no available literature on their arrival. As likely exotic species, particular care would have to be taken in any potential future development as biological control agents for *Polistes* or other invasive wasps.

The method of biocontrol application and how the agent will disperse through the host population is another significant factor, especially in the case of non-mobile organisms such as fungi. The ability of *Beauveria* species to infect hosts via the oral tract could be beneficial in the delivery of propagules in a bait or lure, as shown in bioassays in chapter three. Toxic protein baits have proven highly successful in the management of *Vespula* wasps (Beggs et al., 2011; Lester et al., 2013; Lester & Beggs, 2019), as unlike *Polistes* species, *Vespula* spp. will scavenge for protein food sources (Richter, 2000; Toft & Harris, 2004). A previous study found success

with incorporation of *Metarhizzium* and *Beauveria* conidia into protein baits for *Vespula* wasps (Brownbridge et al., 2009). Chemical lures may have potential as species-specific attractants for non-scavenging wasps such as *P. chinensis* (Elmquist & Landolt, 2018; Landolt & Zhang, 2016), though none have yet been developed for this species, or any *Polistes*, to my knowledge. The combination of a species-specific lure with a toxin or pathogen in a control agent could provide a useful tool in population management with limitation of non-target effects, which is of a major concern regarding the use of broad-spectrum pesticides (Pisa et al., 2015; Sakamoto et al., 2019).

A biocontrol pathogen ideally should proliferate through a colony once introduced for effective knock-down of the colony and suppression of the population. A previous field trial of entomopathogenic fungi controls for *Vespula* wasps noted that introduction of a pathogen to the nest via a few infected individuals is not necessarily enough to induce an epizootic in the colony, likely due to colony hygiene behaviours (Harcourt, 2002). Due to the division of foraging labour within *Polistes* colonies (Daugherty et al., 2011; Tsuchida, 1991), fungal conidia ideally should be able to be reliably passed on from foraging to non-foraging wasps, and to developing brood, to promote pathogen transfer. Harris et al. (2000) showed that this transfer is possible in *Vespula* sp. but that oral inoculation of wasps infected did not significantly increase mortality rates in non-inoculated adults or larvae. The hypothesised adaptations of *O. humbertii* for dispersal from host to host must in many circumstances be able to overcome colony hygienic behaviours against fungal pathogens. During data collection in the field, at least two of the 18 nests found with hosts of *O. humbertii* had evidence of daughter wasps as well as the foundress wasp succumbing to infection. All infected individuals from all nests were found with cadavers firmly grasping the nest comb, while one *B. malawiensis* host was found on the ground approximately 1 m from a nest with several adults. These observations may suggest adaptations of *O. humbertii* to optimise dispersal between nestmates in social wasps, or perhaps even wasps attempting intercolonial cannibalism of the affected nest (Kasuya et al., 1980), as hypothesised in other studies (Sobczak & Somavilla, 2020; Somavilla et al., 2020a).

The logistical aspects of how a fungal biocontrol agent may be mass cultured, stored, and applied must also be addressed. The benefits of *Beauveria* spp. in this case is that they are easily cultured and mass produced, although due to the hydrophobic coating of the conidia must be suspended in oils or hydrophilous solutions for liquid-spray applications (Behle & Birthisel, 2014; Mascarin & Jaronski, 2016). On the other hand, the hydrophilic mucous-coated

conidia of species such as *O. humbertii* can be easily suspended in aqueous solutions, but many hirsutelloid species have been noted as difficult to culture in vitro, having slow growth and requiring highly specific abiotic conditions (Boucias et al., 2007; Wraight et al., 2018). Once again, specific isolates or strains being cultured is also important, due to the high level of divergence even within a single species (Li et al., 2013; Sung et al., 2007).

Finally, the most effective use of fungal pathogens as biocontrols may be as part of an integrated pest management (IPM) strategy in combination with other control methods. Agents that could potentially reduce colony hygienic behaviour or otherwise promote infection could improve efficacy of pathogenic controls (Lacey et al., 2015). Many pathogens or parasites are opportunistic or can be vectored by other organisms, and pathogen and parasite loads may be higher in already weakened insect colonies where other pathogens are already present (Laine & Mäkinen, 2018; Quinn et al., 2018).

4.4 Conclusions, implications for management, and options for future research

In my surveys of *Polistes chinensis* colonies in the Onetahua Nature Reserve and diet analysis, I found no direct evidence that this population currently features higher colony survival rates or densities than found in other studied areas, or that the prey community includes species of known conservation importance. However, the significance of Onetahua Nature Reserve as a site of important invertebrate biodiversity cannot be understated. The metabarcoding method used for diet analysis was not able to identify prey species absent from the barcode reference library, and thus likely missed rare or highly endemic species. While wasp predation of pest species such as the white cabbage butterfly (*Pieris rapae*) has been noted as a positive factor in agricultural systems and other built-up areas (Clapperton, 1999), reserve land and rural coastal habitats such as Onetahua likely exhibit an insect community with a higher proportion of native and endemic species (Patrick & Dugdale, 2000; Patrick & Patrick, 2019; Trewick & Morgan-Richards, 2020). Any positive effects of invasive species control that paper wasp predation might confer in such habitats is probably outweighed by effects on native species.

Considering these factors, I recommend that repeated surveys of wasp and prey species relative abundances be carried out on Onetahua. Survey data can easily be gathered by volunteers and citizen scientists, producing valuable ecological datasets, especially when standardised monitoring techniques are used (Le Féon et al., 2016; Montgomery et al., 2021). With a suitable sampling scheme and minimal training, volunteers can produce observational

data on insect populations equivalent to that of expert entomologists (Kremen et al., 2011). During February 2022, I trialled three potential surveying techniques that may prove suitable for monitoring paper wasp and copper butterfly (*Lycaena* spp.) abundance and could be carried out by volunteers or citizen scientists with minimal training (see Appendix C). Data from these tests and the colony density and abundance estimates generated in Chapter 2 could be used as a baseline for building long-term population datasets. Such datasets can be invaluable for determining population-level impacts of predation on prey species, as exemplified for *P. dominula* predation effects by McGruddy et al. (2021b).

Engagement of citizen scientists and volunteers in surveys like these proposed would also build on the positive engagement of the local community in conservation projects. Participants would also gain increased awareness of invasive paper wasps and their ecological impacts, as well as increased vigilance for wasp nests and other invasive species in other areas. With *Polistes dominula* rapidly expanding their range in Aotearoa, awareness of the possibility for and likely dispersal pathways of *Polistes dominula* to the Nature Reserve should be maintained. Due to the superficial similarity between *P. chinensis* and *P. dominula* (M.P.I, 2016), it is likely that individuals or nests of the latter could be misidentified as the former during the recommended abundance surveys. As a synanthropic species, this wasp commonly nests in or on man-made structures such as the eaves of buildings (Benadé et al., 2014; Roets et al., 2019). To this end, I would highly recommend regular checking of buildings, fences, vehicles, and other anthropogenic materials or potential shelters in Pūponga, Whararikі, and other nearby areas with high human traffic rates, for the presence of *P. dominula* wasps and nests.

Finally, biological control is currently considered the most viable avenue for further research into control methods for *Polistes* wasps (Brown, 2021), potentially integrated as part of a broader management plan (Lester et al., 2013). While there would likely be several challenges in the development and application of such methods, entomopathogenic fungi may be considered as potential candidates for *Polistes* biocontrol agents in Aotearoa and around the world.

Appendix A: Chapter 2 supplementary material

Bioprojects for metabarcoding sequence data:

Onetahua: https://www.ncbi.nlm.nih.gov/bioproject/PRJNA856715

Nelson: https://www.ncbi.nlm.nih.gov/bioproject/PRJNA856051

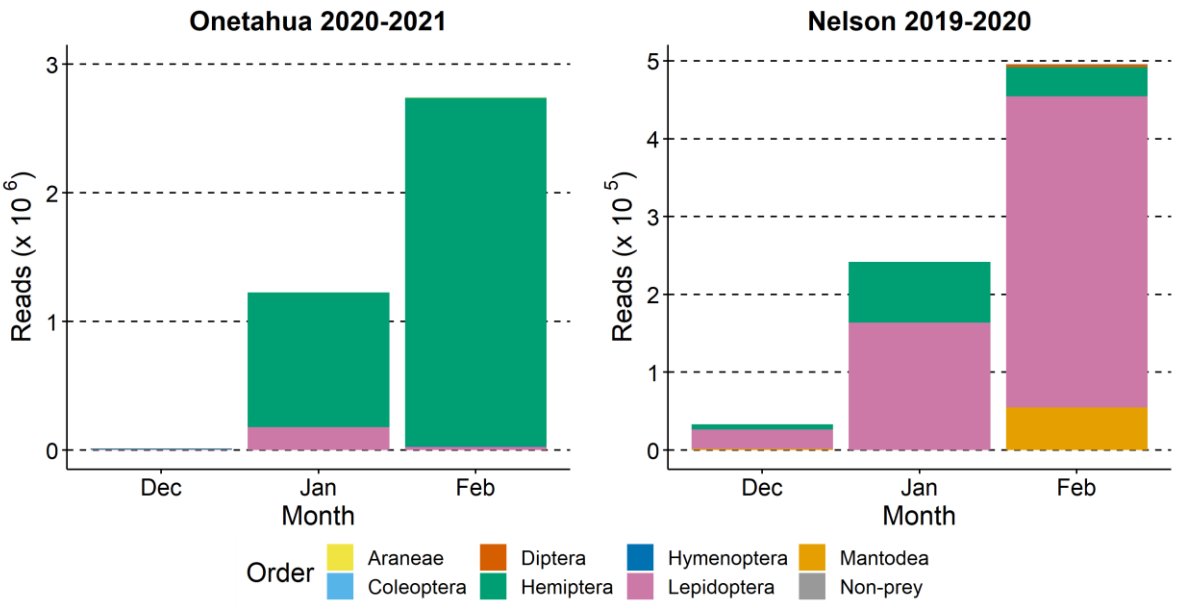


Figure A.1 Read abundance per month for each site dataset, and corresponding taxonomic order, across all samples. The data used to construct these plots includes all assigned taxa, including non-target taxa and assigned species that are recorded absent in Aotearoa and therefore not identifiable with precision to genus or species.

Figure A.2 Individuals of two *Lycaena* spp. caught on Onetahua in February 2021. A-C) Individual tentatively identified as a male of the *L. salustius* complex. D,E) Individual tentatively identified as a female of the *L. feredayi* complex. Identifications based on wing markings and abdomen shapes described in (Gibbs, 1980a, 1980b). Scale bars ~1 cm. Photographs: Aiden Reason.

Table A.1 Numbers of *Polistes chinensis* larvae gut DNA samples collected in each month from Onetahua, and numbers of samples retained after PCR quality control (QC), and after data cleaning and filtering processes (Filtered). PCR quality control was carried out by Custom Science (Auckland, New Zealand). Data cleaning and filtering was done post-taxonomic assignment to remove poorly aligned sequences and reduce the number of false positives.

	Dec 2020	Jan 2021	Feb 2021	**Total**
Sample collection				
Nests sampled	16	16	17	49
Larvae collected	32	32	34	98
PCR QC				
Nest samples	15	13	16	44
Larvae samples	25	20	27	72
Filtered				
Nest samples	13	13	16	42
Larvae samples	19	20	27	66

Table A.2 All species/mOTUs identified in the *P. chinensis* diet analysis, grouped taxonomically. The number of samples that an assigned species or mOTU was retrieved from is stated, as well as the months in which samples containing these taxa were collected. GenBank reference accession numbers for each sequence used in taxonomic assignment for each species/mOTU are given, including multiples for the same species/mOTU.

Order/Family	Species/mOTU	Biostatus	No. samples	Month	GenBank no.
Araneae					
Araneidae	*Argiope protensa*	Native	6	Dec; Jan; Feb	KJ957983.1
Linyphiidae	*Tenuiphantes tenuis*	Exotic	4	Feb	KC244266.1, KC244263.1, MW998738.1, MW998746.1
Pisauridae	*Dolomedes* sp. CG96	Native	10	Jan	KY017872.1
Tetragnathidae	*Leucauge dromedaria*	Native	10	Dec; Jan	HQ986505.1
Theridiidae	*Cryptachaea blattea*	Exotic	5	Feb	JN859116.1
Diptera					
Calliphoridae	Calliphoridae 1 [2]		2	Jan	MN537823.1
Culicidae	*Culex pipiens*	Exotic	1	Jan	MK714005.1, MK402879.1, MN299023.1
Syrphidae	*Melanostoma fasciatum*	Endemic	11	Feb	KY842329.1
Hemiptera					
Aphrophoridae	*Philaenus spumarius*	Exotic	29	Dec; Jan; Feb	MG406114.1, KR042931.1, KR918067.1, MF830242.1, MF838291.1, MG397889.1, MG401535.1, MG405830.1, MG406879.1, MK188565.1, MZ634082.
Cicadidae	*Kikihia muta*	Endemic	6	Feb	MG737737.1, MZ470304.1
	Kikihia subalpina	Endemic	21	Feb	MZ470285.1
	Rhodopsalta cruentata	Endemic	54	Dec; Jan; Feb	MZ470326.1, MZ470286.1, MZ470310.1, MZ470321.1, MZ470322.1, MZ470328.1, MZ470333.1, MZ470334.1, MZ470340.1, MZ470342.1, MZ470343.1
	Rhodopsalta leptomera	Endemic	48	Jan; Feb	MZ470341.1, MZ470346.1, MZ470348.1
	Cicadidae 1 [2]		50	Dec; Jan; Feb	MN136283.1
Miridae	*Sidnia kinbergi*	Exotic	4	Feb	MH359357.1
	Stenotus binotatus	Exotic	4	Dec	MF938990.1, KM022221.1
Pentatomidae	*Nezara viridula*	Exotic	7	Feb	MT179300.1
Hymenoptera					

Braconidae	*Meteorus pulchricornis*		Exotic	38	Dec; Jan; Feb	MK736041.1, LC467412.1, LC467497.1
Lepidoptera						
Batrachedridae	*Batrachedra* sp.	[1]	Native/ Endemic	14	Jan; Feb	MG362932.1
Coleophoridae	*Coleophora* sp. PW154		Exotic	8	Feb	KF153714.1
Crambidae	*Eudonia* sp.	[1]	Native	4	Feb	MG464509.1
	Herpetogramma thestealis		Exotic	3	Jan	KT145294.1
	Uresiphita ornithopteralis		Exotic	7	Feb	KF153895.1
	Crambidae 1	[2]		4	Jan	HM906371.1
	Crambidae 2	[2]		6	Jan	KF391486.1
	Crambidae 3	[2]		10	Jan	KT145294.1, MG359707.1
Depressariidae	*Agonopterix* sp. 1	[1]	Exotic *	17	Jan; Feb	MN968443.1
	Agonopterix sp. 2	[1]	Exotic *	45	Dec; Jan; Feb	MN968488.1, MG358095.1
	Agonopterix sp. 3	[1]	Exotic *	15	Jan; Feb	GU092197.1, MG362657.1, MG470368.1
Erebidae	Erebidae 1	[2]		24	Dec; Jan; Feb	KF388809.1
	Erebidae 2	[2]		2	Jan	KR070795.1
	Erebidae 3	[2]		10	Dec	HQ569693.1
	Erebidae 4	[2]		13	Jan; Feb	KJ013103.1
	Erebidae 5	[2]		6	Dec; Feb	MK158530.1
	Erebidae 6	[2]		17	Dec; Feb	MK158531.1
	Erebidae 7	[2]		4	Feb	MF129292.1
	Erebidae 8	[2]		2	Feb	HQ921670.1
	Erebidae 9	[2]		26	Jan; Feb	HQ950453.1
	Erebidae 10	[2]		8	Feb	JF840288.1
	Erebidae 11	[2]		3	Jan	MK767721.1
	Erebidae 12	[2]		5	Feb	MF923529.1
	Erebidae 13	[2]		4	Dec	MF128271.1, MF132828.1
Gelechiidae	*Monochroa* sp.	[1]	Exotic	1	Jan	JN262188.1
	Gelechiidae 1	[2]		13	Jan	MN805651.1
	Gelechiidae 2	[2]		4	Jan	KF392691.1
	Gelechiidae 3	[2]		10	Jan	MG357225.1, GU691960.1, MG357249.1, MG358955.1, MG364131.1, MG365083.1
	Gelechiidae 4	[2]		1	Dec	HM381409.1

	Gelechiidae 5	[2]		18	Jan	KT146609.1, KT134361.1
Geometridae	*Chloroclystis filata*		Exotic	8	Dec	KF392003.1
	Cyclophora sp.	[1]	Exotic	5	Jan	MG364545.1
	Declana leptomera		Endemic	5	Dec; Jan	KF153722.1
	Pasiphila sp.	[1]	Native	8	Dec; Feb	OK156362.1
	Poecilasthena pulchraria		Native	3	Feb	KF390337.1
	Scopula sp.	[1]	Native	11	Jan	KF390568.1
	Geometridae 1	[2]		6	Jan	JN267272.1, KF388504.1
	Geometridae 2	[2]		1	Jan	MK739633.1
	Geometridae 3	[2]		7	Jan	KR070768.1
	Geometridae 4	[2]		1	Jan	MK739267.1
	Geometridae 5	[2]		29	Dec; Jan; Feb	KX951539.1
	Geometridae 6	[2]		9	Jan	KX071620.1
	Geometridae 7	[2]		5	Dec; Jan; Feb	KU874906.1
	Geometridae 8	[2]		1	Jan	MG572815.1
	Geometridae 9	[2]		9	Jan	MK019586.1
	Geometridae 10	[2]		2	Jan	KF392724.1
	Geometridae 11	[2]		2	Dec	KX045733.1
	Geometridae 12	[2]		3	Jan	JQ577367.1
	Geometridae 13	[2]		3	Jan	MW479743.1
	Geometridae 14	[2]		3	Jan	MW479569.1
	Geometridae 15	[2]		10	Dec; Jan; Feb	JQ564778.1
	Geometridae 16	[2]		1	Jan	JF846977.1
	Geometridae 17	[2]		2	Dec	KM541961.1
	Geometridae 18	[2]		8	Jan	MK758426.1
Lycaenidae	*Lycaena* sp.	[1]	Native	2	Feb	MN829492.1
	Lycaena salustius		Native	38	Dec; Jan; Feb	KF153772.1, KF153773.1
Noctuidae	*Agrotis munda*		Exotic	6	Dec	KF394254.1
	Athetis tenuis		Exotic	2	Feb	KX534776.1
	Helicoverpa armigera		Exotic	4	Dec	KM275041.1, KY411201.1, LC548613.1
	Noctuidae 1	[2]		2	Feb	MN715147.1
	Noctuidae 2	[2]		3	Dec	JQ557400.1
	Noctuidae 3	[2]		2	Feb	HM425841.1

	Noctuidae 4	[2]		45	Dec; Jan; Feb	HM386978.1, KX043990.1, KX047455.1
	Noctuidae 5	[2]		2	Jan	KM572557.1, KX048029.1
	Noctuidae 6	[2]		3	Jan	JN270330.1
	Noctuidae 7	[2]		13	Feb	JQ556702.1
	Noctuidae 8	[2]		4	Jan	KJ390286.1
	Noctuidae 9	[2]		5	Feb	HQ583756.1
Oecophoridae	*Eulechria* sp. 1	[1]	Native	6	Jan; Feb	KF401039.1
	Eulechria sp. 2	[1]	Native	4	Jan	KF405425.1
	Oecophoridae 1	[2]		7	Jan	KF405058.1, KF398610.1
	Oecophoridae 2	[2]		28	Dec; Jan; Feb	KX044079.1
	Oecophoridae 3	[2]		8	Jan	KF397533.1
	Oecophoridae 4	[2]		6	Jan	KF404374.1
	Oecophoridae 5	[2]		12	Jan	KF402960.1
	Oecophoridae 6	[2]		3	Jan	KF397883.1
	Oecophoridae 7	[2]		7	Jan	KF402208.1
Saturniidae	Saturniidae 1	[2]		10	Dec; Feb	JX216126.1, GU663268.1
Tortricidae	*Epiphyas postvittana*		Exotic	16	Dec; Feb	HM346384.1, HM346404.1, HM346409.1, HM346473.1, MG851790.1
	Holocola zopherana		Native	5	Jan	KF399527.1
	Tortricidae 1	[2]		2	Jan	JQ568838.1
	Tortricidae 2	[2]		1	Jan	KC430500.1
	Tortricidae 3	[2]		9	Dec; Jan; Feb	KF403393.1
	Tortricidae 4	[2]		3	Dec:Jan	KJ592405.1, KJ592408.1
	Tortricidae 5	[2]		10	Jan; Feb	KF396664.1, KF403756.1
Unknown family A	Unknown family A sp. 1	[3]		15	Jan; Feb	KT146446.1, KT131177.1, MG508640.1
	Unknown family A sp. 2	[3]		10	Jan	MG505595.1
	Unknown family A sp. 3	[3]		4	Jan	KR451300.1
Unknown family B	Unknown family B sp. 1	[3]		8	Jan; Feb	MW031134.1
Unknown family C	Unknown family C sp. 1	[3]		1	Jan	AB915691.1
Unknown family D	Unknown family D sp. 1	[3]		2	Feb	MT358269.1

Peronosporales					
Peronosporaceae	*Peronospora* sp.	[1,4]	7	Jan	KJ654086.1
Pucciniales					
Pucciniaceae	*Puccinia graminis*	[4]	4	Jan	HQ317629.1
Caryophyllales					
Polygonaceae	*Fagopyrum esculentum*	[4]	17	Dec; Feb	MT318701.1
Gentianales					
Rubiaceae	*Rubiaceae* sp.	[2,4]	2	Dec	KY637942.1

[1] The closest species-level match is not recorded present in New Zealand

[2] The closest genus-level match is not recorded present in New Zealand

[3] The closest family-level match is not recorded present in Zealand

[4] Non-target taxa

* Exotic species intentionally released for biological control of a pest species

Appendix B: Chapter 3 supplementary material

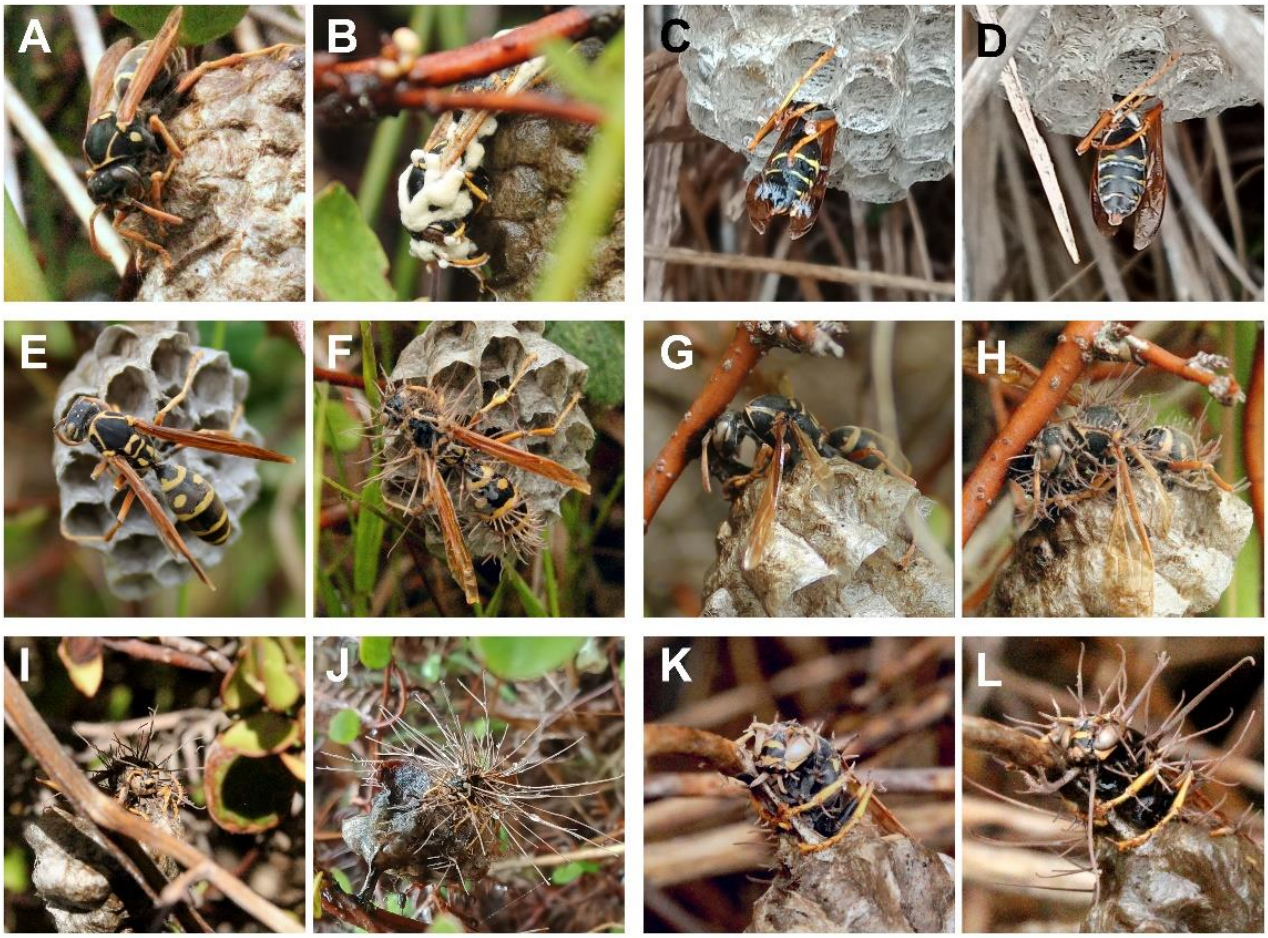


Figure B.1 Development of conidiogenesis on *P. chinensis* cadavers over time. Six specimens of infected *P. chinensis* wasp cadavers with external conidiogenous bodies developing over time, photographed in situ on Farewell Spit over the 2021 – 2022 austral summer. Adjacent photographs are of the same mycosed specimen, with photos on the right side of each pair taken one to two weeks after the first. **A,B)** *B. malawiensis*, cadaver had pale ocelli and brown eyes in A, lightening slightly after conidiogenesis in B. **C,D)** *B. malawiensis*, having killed its host while the wasp was partially inside a comb cell; yellow colouration on the cadaver in C becoming dull in D, and white mycelium emerging from between abdominal sternites. **E – L)** *O. humbertii*, all host specimens with characteristically silver-coloured eyes and ocelli, and dull cuticle. **E,F)** and **G,H)** show development from having no external conidiogenous bodies to mature synnemata; **I,J)** and **K,L)** show development from young to mature synnemata. Host wasps are approximately 2 cm long. Photographs: Aiden Reason.

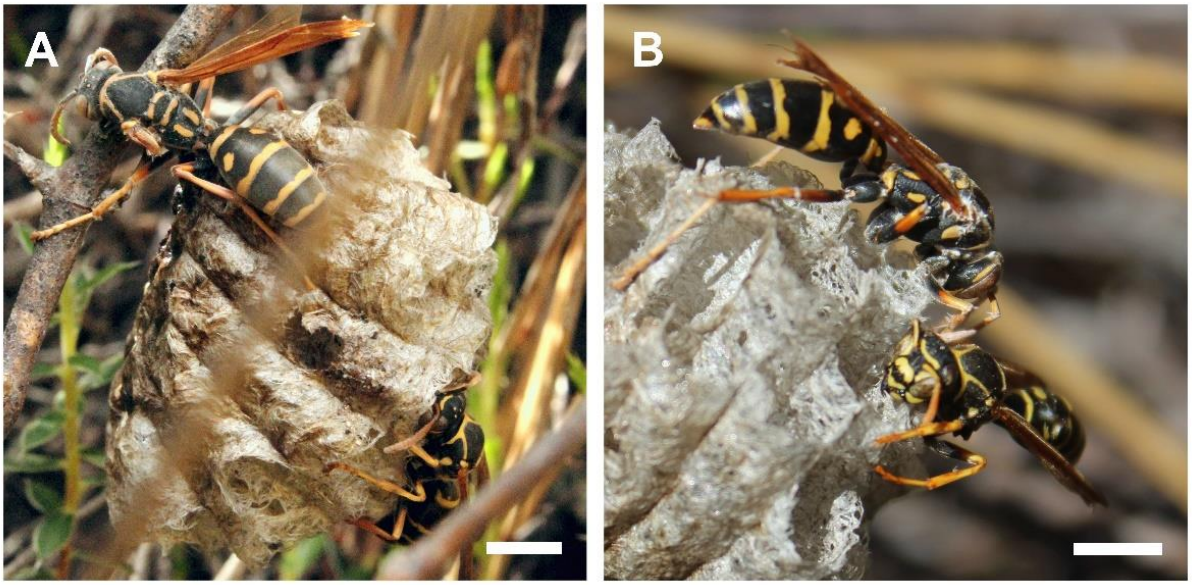


Figure B.2 Potential disease transmission between nest mates. Specimens of *P. chinensis* nests wherein the foundress wasp is dead and infected with *O. humbertii*, and an emerged adult wasp cadaver on the same nest also shows evidence of fungal infection. It is possible that the daughter wasp in each case contracted their infections from the respective mycosed foundress, but we cannot confirm this. **A)** This nest was discovered when the foundress (upper left) and daughter wasp (lower right) were both already dead and mycosed. The foundress cadaver was in the early stages of synnemata development. Photographed *in situ* on Farewell Spit, Jan 2022. **B)** This nest was discovered in January 2021 with the foundress (upper left) dead and the cadaver in the early stages of synnemata development. At this time the daughter wasp was alive and appeared healthy. The nest also housed eggs, three larvae, and three capped pupal cells. We revisited the nest two weeks later, to find the daughter wasp dead with fungal infection, and the immature brood gone or undeveloped. Photographed *in situ* on Farewell Spit, Feb 2021. Photographs: Aiden Reason.

Figure B.3 *Polistes dominula* cadaver with fungal infection, evidenced by silver-coloured eyes and ocelli, and a pale tomentum on the left antennae. The pose of the wasp body shows a firm grasp on a dry grass stem with the legs and mandibles, which is consistent with the pre-mortem behaviour observed from similar cadavers by Somavilla et al. (2020a) and Sobzack *et al.* (2020) in swarm-founding Polistine hosts. Photographed *in situ* in the Nelson region, New Zealand, Nov 2021. Scale bars = ~0.5 cm. Photographs: Mariana Bulgarella.

Table B.1 Sample data for collected nests and individual wasp cadavers with evidence of entomogenous fungal infection. Where conidiogenesis was never observed in a specimen, fungi species is listed as "unknown." Cells is the number of cells in the nest comb. Immatures includes larvae and capped cells with pupae.

Nest ID	Cells	Fungi sp.	Immatures	Live adults	Date found
254	32	*B. malawiensis*			12 Jan '21
367-B	*n/a*	"			10 Feb '21
BW1	17	"			18 Dec '20
241	20	*O. humbertii*			8 Jan '21
250	29	"			8 Jan '21
270	27	"	6	1*	15 Jan '21
271	18	"			15 Jan '21
273	28	"			15 Jan '21
275	15	"			15 Jan '21
326	15	"			4 Feb '21
346	10	"			5 Feb '21
379	29	"			12 Feb '21
SW1	10	"			18 Dec '20
136	35	unknown			**31 Dec '20
295	49	"	21	5	27 Jan '21
297	64	"	20	5	29 Jan '21
302	30	"	10	2	29 Jan '21
388	26	"			15 Jan '21

* Upon next visiting nest 270, this adult was dead on the nest also.

** Date that the foundress of this nest was found dead; the foundress was alive at previous visit on 17 Dec, with 12 immatures.

Appendix C: Testing methods for surveying the relative abundances of paper wasps and prey species

C.1 Background

Populations can differ greatly year to year as a product of natural variation, abiotic factors, and the life history of a species. Long-term datasets of at least five to ten years are typically required to determine population trends with a sufficient level of statistical power (Rueda-Cediel et al., 2015; White, 2018). Availability of established population data prior to management implementation is essential for the evaluation of management efficacy and for informing management adaptation (Armstrong et al., 2007). Paper wasp density and abundance data gathered in this study will ideally form a baseline for ongoing population monitoring, so that a long-term dataset may be accumulated on the relative abundances of *Polistes* wasps and key prey species on Onetahua. To this end, I aimed to determine a survey method or combination of methods for measuring *Polistes* wasp abundance that met the following criteria:

1. Able to be standardised and easily replicated.
2. Able to be carried out by conservation volunteers or citizen scientists with limited training.
3. Practical to implement within the limitations of the Farewell Spit Nature Reserve.
4. Produces relative abundance data that is proportional to the known or estimated actual wasp abundance of an area.
5. Can be adapted or supplemented to simultaneously monitor other species of interest.

C.2 Methods

During the second summer of data collection on Onetahua (November 2021 – February 2022) I tested three methods of surveying for paper wasp relative abundances based on outlines for standardised invertebrate monitoring practices by Montgomery et al. (2021). These methods were yellow pan trap (YPT) surveys, point count surveys, and distance sampling using line transects (Thomas et al., 2010). Survey locations are mapped in Fig. C.1. The data collected from surveys of 0.5 ha sample plots and extrapolated colony density estimates (outlined in

Chapter 2) were used as a benchmark against which to compare the data from these survey trials. The goal of these comparisons was to select the survey method with the most desirable balance of accuracy and ease of use.

Figure C.1 Satellite map of Onetahua marking survey locations. Yellow points are locations of yellow pan traps; pink points are 10-minute point count locations; green bars are transect lines for Distance sampling. Aerial imagery downloaded from https://data.linz.govt.nz/ and compiled in QGIS 3.25. Map scale: 1:40000.

C.2.1 Yellow pan traps

Pan-trapping is an insect monitoring method commonly used for sampling pollinator species, especially winged Hymenoptera and Diptera (Abrahamczyk et al., 2010; Montgomery et al., 2021). Traps consist of a coloured plastic pan, typically yellow, white, or blue, semi-filled with water and a small amount of detergent to break water tension, to which a scent lure is sometimes added (Montgomery et al., 2021; Padrón et al., 2021). Pollinator insects are attracted to the pan colour and/or scent lure, become trapped in the liquid, and can be later collected. Yellow pan traps have been effectively used to sample *Polistes versicolor* in the Galapagos Islands (Parent et al., 2020) and Hymenopterans generally (Buffington et al., 2021; Westphal et al., 2008). The preferred colour of trap for sampling various species is the subject of some debate, and can vary substantially with habitat (Saunders & Luck, 2013). However, yellow is generally considered the most effective option for most Hymenopteran species, including those within the family Vespidae (Abrahamczyk et al., 2010; Padrón et al., 2021).

Pan traps were set out at seven locations on Onetahua between 18 December 2021 and 7 February 2022 in the centre of the 0.5 ha sample plots described in section 2.3.1, and at two additional locations near the landmarks known as “Gull Gap” and “Mullet Creek” (Petyt, 1999) (Fig. C.1). Yellow plastic fuel canisters, cut in half lengthways, with dimensions 250 x 350 x 150 mm and maximum capacity 10 L, were used for the traps (Fig. C.2). Pans set out in December were filled with 4.5 L tap water and 25 mL unscented liquid detergent. The volume of water for

pans set out in January and February was increased to 5.25 L due to increased evaporation rates. One half of an overripe banana was placed half submerged in each trap as a scent lure (Lester, 2021; personal communication). The duration that traps were left in the field varied, as initial tests in which traps were left for 1-2 days collected no paper wasps. Traps set out later in the season were left for 6-7 days of suitable weather conditions for wasp foraging. The metadata collected for each trap deployment included the location, surrounding habitat and vegetation, and the phenology of the dominant plant species nearby (e.g. flowering, fruiting), as suggested by Montgomery et al. (2021). When each trap was collected, all *P. chinensis* in the traps were counted and manually removed with forceps into a container with 90% ethanol. All non-target organisms were then extracted using a strainer, placed in a container with 90% ethanol, and stored at -18° C for eventual identification. The final volume of water in each pan was recorded to track evaporation rates (Montgomery et al., 2021).

Figure C.2 Photographs of a newly placed pan trap and surrounding location on Onetahua, facing each cardinal direction (left to right = clockwise north to west), per recommendations for standardisation of recorded metadata by (Montgomery et al., 2021). The 10 L plastic pans were filled with 4.5 – 5.25 L tap water and 25 mL unscented detergent, with half of an over-ripe banana as a scent lure. Photographs: Aiden Reason.

C.2.2 Point counts

Counting observations of a subject animal or animals within a set time on a point-transect is a commonly used method for monitoring birds (Darras et al., 2018), and has also been used to survey insects (Schmack et al., 2020). It may be a prudent method to use as an alternative to

line transects in landscapes with challenging terrain or vulnerable vegetation, to avoid repeated trampling of the same routes (Montgomery et al., 2021).

Fourteen point locations were randomly placed in QGIS 3.25 (QGIS.org, 2021) within the total vegetated area of Onetahua, as approximated from satellite imagery, per the sampling method described in Schmack et al. (2020). These points were then located in the field via GPS. Where a placed point was not practically accessible due to being within an area of swamp or very dense shrubs, the closest location that was reasonably accessible was used instead.

Observations for each point count were taken over 10 minutes by two observers. All sightings of *Polistes chinensis* and copper butterflies (*Lycaena* spp.) were recorded as tallies of each species, with observers communicating throughout to avoid individual sightings being recorded twice. The sampling area was restricted to all visible area within an approximately 15 m radius of the observers, as this was approximately the maximum distance that an individual of *P. chinensis* in flight could be confidently identified, although copper butterflies could be identified from a greater distance. Paper wasps can be differentiated from other flying insects at a distance by characteristic flight patterns (Schmack et al., 2020), and copper butterflies are (*Lycaena* spp.) easily identified by colour, size, and flight motion (Gillespie & Wratten, 2012). Metadata recorded included the time of day, general weather conditions, point location, surrounding habitat and vegetation including phenology (Montgomery et al., 2021). Wind speed and air temperature were automatically recorded at regular intervals during observation using a handheld anemometer (Benetech air flow anemometer GM8902).

I used negative binomial regression models in R 4.1.1 with the package *MASS* (R Core Team, 2020; Venables & Ripley, 2002), to explore the main factors influencing copper butterfly observations in point counts. The explanatory variables for butterfly observations entered into these models were the date and time of surveying, average wind speed (m/s) and average air temperature (°C) while surveying, and the number of wasps observed, per point count.

C.2.3 Distance sampling

Distance sampling consists of one or more observers counting subjects or clusters of subjects, usually from a line transect, with an estimate of the perpendicular distance of the subject or cluster from the transect line. Observational data can then be analysed using Distance software (Thomas et al., 2010), which models the probability that an observer will detect a subject at any given distance. This model can then produce an estimate of subject density per unit area.

Twenty line transects each 100 m in length were manually placed in QGIS 3.25 to align with previously recorded GPS track data in relatively linear sections. Transects were spaced at least 100 m apart in an attempt to ensure independence, as maximum foraging distance for *P. chinensis* adults is approximately 70 m, or 120 m^2 (Suzuki, 1978). Due to the high degree of heterogeneity in the Onetahua landscape and logistical constraints of time and transportation, random or stratified placement of transects was impractical. Instead, transects were clustered in four main locations with reasonable accessibility (Fig. C.1 and C.3).

Figure C.3 Photographs taken from the same location on Onetahua approximately 10 km from the eastern base, facing east (left) and west (right). There is a range of contiguous dunes several kilometres long beginning about 9 km along the spit and running east against the southern spit edge. The vegetation in this area is short and dry, consisting mostly of rushes, grasses, coastal scrub < 1 m in height, and various herbaceous plants. This vegetation cover makes the landscape comparatively easy to navigate, as well as high-quality habitat for paper wasps. High nest densities were recorded in this area in both study seasons, and it was the chosen location for line transects T6 – T14. Lower-lying areas to the immediate north, and much of the vegetated areas generally, are dominated by salt marsh, mud flats, and dense shrubs. On the right side of the left image, the easternmost end of the spit's inner beach is visible. Photographs: Aiden Reason.

Transects were then located in the field using a GPS. Before starting each transect, metadata including the date, time of day, cloud cover as a percentage, and the overall weather condition was recorded (Montgomery et al., 2021). Transect lines were walked by two observers at approximately a quarter of normal walking speed, each transect taking about 15 minutes to complete. Observations were either individual paper wasps or wasp nests, with the number of adult wasps visible on the nest recorded as a cluster. Wasps on nests were counted without the aid of a mirror, so these counts should be considered conservative estimates, as adults (especially males) will often rest inside cells, where they are often not visible without the

use of a small hand mirror. For each observation I recorded an estimate of the perpendicular distance between the subject and the transect line, to the closest meter.

I analysed the transect data in Distance 7.4 (Thomas et al., 2010). Due to having a high number of detections at a very short distance, I applied a data filter with six interval cut-points up to the maximum recorded distance of 15 m, to improve model fit. I ran models for each combination of key estimators and adjustment terms. The fit of each model was checked using a χ^2 goodness-of-fit test, and the model with the lowest Akaike Information Criterion value (AIC) was selected (Akaike, 1998).

C.3 Results

C.3.1 Yellow pan traps

A total of seven yellow pan traps were deployed and collected. Considering the long periods in which traps were active, very few paper wasps were caught (Fig. C.4, Table C.1). Catch numbers between traps were not particularly consistent with the densities of *Polistes* nests in the vicinity of each trap or with the time of season. An abundance of non-target pollinator fauna from the expected taxonomic orders Hymenoptera and Diptera were caught, as well as a variety of other Arthropods including damselflies, copper butterflies, small moths, grasshoppers, crickets, planthoppers, cicadas, thrips, and arachnids. The integrity of these specimens was unfortunately negatively affected by the long periods of sitting in water. Swelling and distension of the abdomen was observed in most collected species, though paper wasps could still be easily discerned from non-target specimens.

Figure C.4 Yellow pan trap photographed before collection after one week in the field. Traps collected a wide range of non-target arthropods, including expected pollinators such as flies, butterflies, and native wasps, but also damselflies, cicadas, plant-hoppers, grasshoppers, and arachnids.

Table C.1. Yellow pan trap catches of *Polistes chinensis* wasps, with the dates during which each trap was active. 'Degree days' (Dd) are the number of full days each trap was set out for that were of suitable weather conditions for wasp activity. Maximum wind (m/s), maximum temperature (°C), and precipitation (mm) for the deployed period were taken from climate data from the Farewell Spit weather station, accessed through *CliFlo* (NIWA, 2022).

Location	Dates	Dd	Max wind	Max temp.	Precipit.	*P. chinensis*
P1	20/01/22 - 26/01/22	4	12.1	25.8	12.6	7
P2	18/12/21 - 20/12/21	2	17.6	23.4	0.0	0
P3	19/12/21 - 20/12/21	1	17.2	23.5	0.0	0
P4	07/01/22 - 13/01/22	6	15.5	25.7	0.0	5
P5	07/01/22 - 13/01/22	6	15.5	25.7	0.0	6
Gull Gap	26/01/22 - 07/02/22	7	14.3	24.0	126.2	10
Mullet C.	28/01/22 - 07/02/22	6	13.9	23.8	113.8	1

C.3.2 Point counts

Very few wasps were recorded in ten-minute point counts overall. On only three occasions were any wasps recorded, with a maximum count of four wasps at a single point. Copper butterflies were observed much more frequently, although with highly variable counts (Table C.2). Models for copper butterfly observations found average wind speed during counts to be the only measured factor to have a significant effect (P = 0.001; residual deviance = 36.195, DF = 26), but there were likely many interacting factors influencing butterfly abundance and activity.

Table 2. Counts of *Lycaena* butterflies and paper wasps recorded in 10-minute point counts taken at fourteen randomly placed locations within the vegetated areas of Onetahua. Average wind speed (m/s) and average temperature (°C) were calculated from wind speed and temperature readings taken every 3 seconds with a handheld Benetech anemometer.

Point ID	Date	Time	Wind avg.	Temp avg.	*Lycaena sp.*	*P. chinensis*
PC1	18/01/22	9:33	0.51	26.41	33	0
	20/01/22	11:53	2.83	21.57	9	0
	27/01/22	15:30	0.24	29.00	20	0
PC2	18/01/22	10:48	1.95	21.46	47	0
	20/01/22	11:00	2.27	22.10	25	0
	27/01/22	14:35	1.73	22.50	12	0
PC3	18/01/22	14:20	1.07	23.13	42	0
	27/01/22	13:25	0.95	24.70	42	0
PC4	24/01/22	16:22	1.61	25.00	25	0
PC5	24/01/22	14:55	1.10	27.20	5	1
PC6	24/01/22	13:35	0.73	25.00	9	4

PC7	24/01/22	11:35	0.64	25.50	4	0
PC8	24/01/22	10:25	2.83	23.00	0	0
PC9	28/01/22	12:20	2.44	26.60	4	0
PC10	28/01/22	11:35	2.38	23.20	9	3
PC11	27/01/22	10:40	0.54	24.50	37	0
PC12	27/01/22	9:30	0.60	26.50	45	0
PC13	24/01/22	8:00	0.30	20.80	0	0
PC14	20/01/22	17:40	3.03	21.66	1	0
	24/01/22	8:50	2.07	21.60	5	0

C.3.3 Distance sampling

Twenty line transect surveys were completed, with 169 wasp observations in total (Table C.3). Thirty-one of these observations were of nests with at least one adult wasp, and the maximum number of wasps recorded at a single nest being 30. The remaining observations were of wasps away from nests either in flight or on vegetation, usually as a single individual, although with four instances of two wasps being detected in the same place at once.

Table C.3 Total number of wasps observed during each transect survey, and associated metadata. Time is time beginning each survey, and cloud cover was a visual estimate taken at this time. Wind gust (m/s) and temperature (°C) data obtained from *CliFlo* (NIWA, 2022).

Transect	Date	Time	Cloud (%)	Wind gusts	Day max temp.	Total wasps
T1	16/02/22	12:21	7	8.8	25.7	7
T2	15/02/22	12:08	45	13.4	23.4	67
T3	15/02/22	11:45	50	13.4	23.4	44
T4	15/02/22	11:05	70	13.4	23.4	16
T5	15/02/22	10:30	90	13.4	23.4	44
T6	14/02/22	17:55	3	9.8	20.5	8
T7	14/02/22	17:35	5	9.8	20.5	27
T8	14/02/22	17:15	3	9.8	20.5	13
T9	14/02/22	17:00	3	9.8	20.5	27
T10	14/02/22	16:33	1	9.8	20.5	15
T11	14/02/22	16:00	1	9.8	20.5	63
T12	14/02/22	15:40	1	9.8	20.5	2
T13	14/02/22	15:15	5	9.8	20.5	14
T14	14/02/22	14:45	35	9.8	20.5	64
T15	16/02/22	16:20	0	8.8	25.7	29
T16	16/02/22	15:31	0	8.8	25.7	3
T17	15/02/22	16:21	40	13.4	23.4	0
T18	15/02/22	15:55	70	13.4	23.4	2
T19	15/02/22	15:20	55	13.4	23.4	27

T20	15/02/22	15:05	55	13.4	23.4	15

In the Distance analysis, a hazard-rate key with cosine adjustment model was selected as the best fitting model for wasp detection probability (P = 0.900; Fig. C.5). Though wasps were detected during sampling to a maximum distance of 15 m, the effective strip width (ESW), or the maximum distance from the transect line at which subjects have an effectively high probability of being detected (Thomas et al., 2010), was estimated to be 3.6 m. The model produced a density estimate of 339 (95% CI: 213, 538) wasps per hectare at the time of surveying, which was between 14 and 16 February.

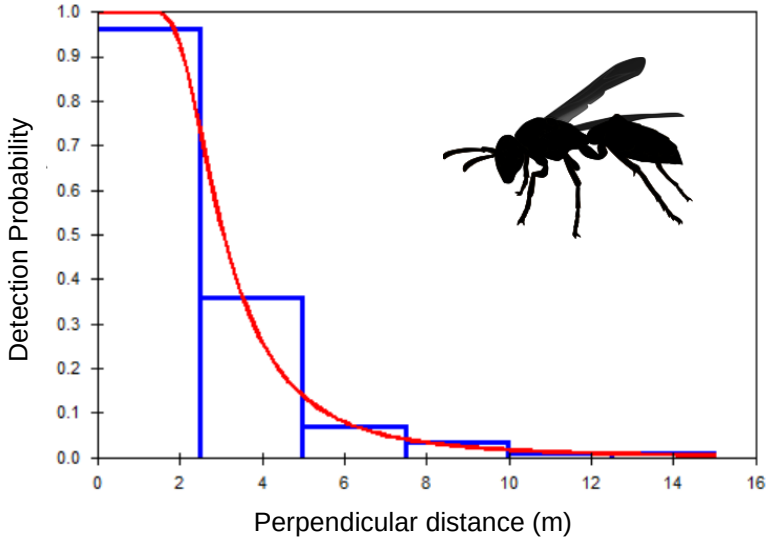


Figure C.5 The detection function for the selected model. Wasps were detected in the field up to 15 m, however for the purposes of this analysis an effective strip width (ESW) of 3.6 m was calculated. The χ^2 goodness-of-fit test found this model to be an appropriate fit (P = 0.900).

C.4 Discussion

I tested three potential methods for surveying paper wasp (and prey species) relative abundances on Onetahua that could be used in future to produce long-term population data for desired species. The methods tested were yellow pan traps, point counts, and distance sampling on 100 m line transects. Each survey method tested had advantages and disadvantages both inherent to the method and in application with the focal species at the study site. These advantages and disadvantages, based on the experience of the observers, are summarised in Table C.1.

Table C.1 Advantages and disadvantages of each survey method tested, based on field observer experience and data gathered. For comparative purposes, the advantages and disadvantages of the sampling method used in Chapter 2, of intensive searching of randomly placed 0.5 ha plots, are also included.

Survey method	Advantages	Disadvantages
Manually searching sample plots	→ High level of accuracy → Data may be gathered on individual colonies	→ Very labour intensive → Frequent and repeated movement through same areas can have damaging effects on vegetation and may influence wasp activity in the area → Ignores foraging wasp activity which may lead to underestimates if nests are missed
Yellow pan traps	→ Easily standardised and requires minimal training to deploy → Allows surveying of a range of insect fauna	→ Equipment is cumbersome and can be challenging to manage on foot → Catch rate for paper wasps is low and traps must be left a long time, leading to degradation of samples → Post-collection processing of samples is labour intensive and requires specialist resources → Relies on indirect counts of foraging wasps only
Point counts	→ Quick and easy to carry out → Effective for surveying of copper butterflies → Could be easily adapted for monitoring of other invertebrates	→ Highly dependent on weather conditions and time of day → Not suitable for low densities or achieving high definition → Relies on counts of foraging wasps only
Distance sampling – line transects	→ Produces wasp density estimates comparable to manual plot searching → Process easily standardised with observer training → Considers both nests and foraging wasps (more robust to weather conditions and nest camouflage) → Easily adapted to also sample other invertebrates	→ Somewhat labour intensive → Difficult to randomise or stratify due to challenges of accessibility and landscape heterogeneity → Counts of foraging wasps still dependent on weather conditions/time of day

The catch rate of paper wasps in pan traps was very low compared to rates found by Parent et al. (2020). Rates were also not very consistent with colony densities recorded from

sample plots (Fig. 2.2; Table 2.1) or with the increase in wasp activity expected over time. These inconsistencies may well be due to the small sample size and high variability among covariates such as deployment dates, times, weather conditions, and locations. However, due to the very low paper wasp catch rate, traps had to be left in the field for up to a week or more, which meant that the size of the trap and water volume had to be great enough to ensure the water did not completely evaporate (or overflow with rainfall) over this time. This factor made the traps cumbersome to deploy and collect, given the necessity of transporting the equipment and water on foot through unpredictable terrain. This practical limitation means that the level of replication potentially needed to overcome extraneous variability in the data would require substantial effort on the part of future surveying volunteers.

Furthermore, the extended period that traps had to be left active for resulted in considerable degradation of specimens. Key diagnostic characters for the identification of some taxonomic groups were lost. While this factor does not inherently hinder paper wasp surveying, it does render this method unsuited to the simultaneous surveying of other Arthropods of interest. Additionally, post-collection processing and identification of entomofauna samples is highly time consuming and requires specialist resources. For high-definition identifications, such as to genus or species level, especially for very small or cryptic fauna, taxonomic expertise is required, and was outside the scope of this study.

Ten-minute wasp counts were easily carried out, with several counts able to be completed consecutively within a relatively short period. The highly heterogeneous nature of the landscape and vegetation on Onetahua likely contributes to significant clustering of nests wasps in preferred nesting locations (Fig. 2.2), and the large proportion of counts performed at which no wasps were observed is consistent with previously recognised levels of occupancy within suitable habitat (Ward & Morgan, 2014). Additionally, wasp foraging activity is considerably variable with the time of day and climatic factors (Clapperton, 1999). Wind speed also had a significant effect on the number of copper butterflies observed.

The distance sampling method yielded wasp observations consistent with the wasp abundance estimates of each area. The line transects had the advantage of “flushing out” insects as observers walked, increasing visibility. Observers are also more likely to come across more foraging wasps with this method as opposed to point counts, as transect lines will likely move through the foraging ranges of several colonies. The consideration of both foraging wasps and whole colonies (as clusters of individuals) also makes this method more robust than

considering either nests or foragers individually. Due to the limited timeframe of the field work and the heterogenous landscape, the transects surveyed in our tests were clustered and deliberately laid on 100 m sections that had been previously visited and could demonstrably be moved through in a straight line, from GPS track data. While a more randomised survey design is usually desirable, for the purposes of relative abundance monitoring, it is more important that the design can consistently record wasp abundance where they are reliably present.

The Distance analysis produced a density estimate of 338 (95% CI: 213, 538) wasps per hectare in late February. The estimate of average colony density at this time was 23 nests per hectare, which would equate to approximately 15 adult wasps on average per active nest, about the expected number for this time. The average number of wasps per nest observed for this time was 11, but as noted previously (section 2.4.1) this is a conservative estimate as it does not account for wasps away from the nest at the time of observation. Thus, I would consider the distance sampling method to be reasonably accurate and sensitive to actual wasp densities when carried out under acceptable conditions.

Barring data analysis using Distance software, this sampling method is overall very similar to standard Pollard surveys (Pollard, 1977) that are well-established for monitoring butterfly abundance. I used a Distance analysis in this case for the purpose of producing a wasp density estimate that could be used to validate the accuracy of this method against the previously calculated colony density estimates. However, for ongoing monitoring, Pollard surveys would likely work just as well. I would recommend that the constraints laid out by Pollard (1977) on when sampling should take place in regard to weather and time of day are also followed for paper wasp sampling.

C.5 Conclusion

Repeated surveying of paper wasp relative abundances on Onetahua and similar areas, alongside surveying of their prey species such as copper butterflies and cicadas, could produce invaluable long-term datasets on populations of these species. Such data may definitively show population-level effects of paper wasp predation on prey species, as found by McGruddy et al. (2021b). Line-transect surveys following the standardised methods of either Pollard surveys (Pollard, 1977) or Distance sampling and analysis (Thomas et al., 2010) would be suitable for this purpose. I believe that these methods should be sensitive enough to detect significant changes in abundances of target species over time. These methods meet the outlined

requirements of being easily standardised and replicated, provided that the location of transects, once established, remains stable, and appropriate metadata is recorded. Location of the transect lines used in this study could be easily found with the use of a handheld GPS, and permanently marked for future years if desired. The software used to analyse the data from these surveys is freely available and analyses may be easily replicated. The sampling method is suitable for the unique Onetahua landscape as transects can be accessed on foot, and materials for recording observations are the only pieces of equipment required. Only minimal training would be required for observers in these surveys to identify the target species, and any conspicuous species could reasonably be surveyed. Other insects easily noticed during surveys were diurnal winged species such as copper and common blue butterflies, red and yellow admirals, cicadas, damselflies, dragonflies, and robber flies. The wasp density estimates produced from the Distance analysis are also consistent with estimates produced from highly intensive surveying of 0.5 ha sample plots and colony monitoring.

Previous studies have shown that long-term datasets produced from transect surveys have been sensitive enough to pick up significant trends in insect populations (Melero et al., 2016; Wepprich et al., 2019). For example, analysis of butterfly abundance surveys by McGruddy et al. (2021b) showed catastrophic declines in several butterfly populations in response to invasive paper wasp predation. I recommend that similar methods be employed on Onetahua in order to inform potential future management implementation for *Polistes* wasps.

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