Bombus spp. in New Zealand

Revising the distribution of *B. hortorum* and investigating the nesting behaviour of *B. terrestris*

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Abstract

Four species of bumblebee have been introduced to New Zealand: *Bombus terrestris, B. ruderatus, B. hortorum* and *B. subterraneous*. They were shipped to the South Island from England in the late 1880s where they quickly established in the Canterbury region. The purpose of their introduction was to facilitate pollination of red clover as very little seed was being set with only the honey bee *Apis mellifera* (also introduced) and New Zealand's small native bees present. Their success in the South Island led to introductions into the North and all but *B. subterraneous* are now present in the North Island.

The bumblebee's ranges and abundances vary between species. *B. terrestris* is ubiquitous in both islands, *B. ruderatus* is found almost everywhere but in lower densities than *B. terrestris, B. hortorum* has been reported as being present only in the South Island, Wellington and Manawatu regions and *B. subterraneous* has a very restricted range in the Canterbury region.

Recent sightings suggested *B. hortorum* was occupying a larger range than reported and this was investigated in the present study. *B. hortorum* and *B. ruderatus* share a cryptic morphology making them almost impossible to differentiate. A new tool has been developed allowing their distinction using a digestion site present in the mitochondrial DNA of *B. hortorum* but not *B. ruderatus*. Specimens from around the country had DNA extracted using the HotSHOT protocol then were subjected to digestion using the Tsp45I enzyme. This led to confirmation that the range of *B. hortorum* does extend into the Waikato region. Further research is needed to determine exactly how far north the species spreads.

Bumblebees are important pollinators of many plants, not just red clover, and the ability to increase population densities in areas growing certain crops is desirable. One way to achieve this is by providing queens with artificial nesting sites known as domiciles. Studies conducted so far on domiciles show large variations in results but most often they are not selected preferentially by queens and do little to increase nest densities, especially for *B. terrestris*. By learning more about what attracts *B. terrestris* queens to natural nest sites the design and placement of domiciles may be altered to encourage higher nest establishment rates.

The present study used random transect walks across field sites in the Netherlands and New Zealand to look for queens exhibiting nest search behaviour. Each time a queen was encountered various pieces of information were recorded about her movements and location. The study culminated with the conclusion that *B. terrestris* queens displayed a preference for searching under trees, more specifically, mixed forest plots in the Netherlands and evergreen trees in New Zealand. They also seemed drawn to areas with moss and leaf litter as the primary ground cover. This information can be applied to domicile design and placement to see if higher nest acceptance rates can be achieved than in previous research.

Also considered as part of this research was the efficacy of radio telemetry tracking of queens to help locate early nests in the wild. Five queens were successfully tagged with 0.2 gram miniaturised radio transmitters in the Netherlands but only one nest site was located. No queens were tagged and tracked in New Zealand. Continued advances in technology relating to radio telemetry may allow the methodology to be more useful in the future.

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"Let us be grateful to the people who make us happy,

they are the charming gardeners who make our souls blossom."

- Marcel Proust

Thesis Structure

This thesis is divided into 5 chapters: a general introduction, three research chapters written in the form of stand-alone papers and a final chapter of general conclusions and recommendations.

Due to presenting chapters two, three and four as separate entities there is some repetition between introductions and conclusions.

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Chapter One

Introduction

Introducing *Bombus* Species to New Zealand

Europeans first colonised New Zealand in the late 18th century and cleared huge areas of native forest to use as farmland. They set out to make New Zealand as productive as their homeland using the skills, tools and various flora and fauna of centuries of agriculture in Europe. By 1890, 2.8 million hectares of New Zealand had been transformed into agricultural grassland (Brooking *et al.*, 2011).

The honey bee, *Apis mellifera*, was imported to provide honey for settlers and it quickly became established in its new habitat in managed and wild hives (Donovan & Macfarlane, 1984). New Zealand's native bees are small in size and their population numbers low, preventing them for easily pollinating the European plants that the settlers began cultivating. Subsequently, honey bees filled another role as essential pollinators (Donovan, 2007). *A. mellifera* was particularly important in white clover crops but due to their size and short tongues they were unable to facilitate pollination of red clover (*Trifolium pratense* L.), which has a deeper corolla length requiring large insects for fertilisation (Palma *et al*, 2005). Red clover is economically important as a key forage crop in agriculture and without sufficient pollination new seeds have to be sown each year incurring large costs to farmers (Gurr, 1974).

In Europe, the niche of red clover pollinator is filled by various species of *Bombus* (Holm, 1966), none of which were present in New Zealand at the time of European settlement. It was decided that the 'humble-bee' must be brought to Aotearoa.

From the 1870s onwards there were quite a few attempts at shipping queen bumblebees from England for release in New Zealand (Dumbleton, 1948). The long sea voyages meant the uplifted queens often did not survive the journey, or those that did were not always in a healthy state. Two particularly successful importations near Christchurch in 1885 and 1906, however, did allow some species of *Bombus* to become established in the South Island (Gurr, 1964). At the time it was not recorded exactly which species were imported and it is likely more than those which eventually became established were introduced (Hopkins, 1914).

It is understood that from as early as 1888 attempts were made to move queens from the South to North Island (Gurr, 1972). The success of several of these introductions lead to a strong presence of *B. terrestris* and *B. ruderatus* in the lower North Island by the time R. A. Cumber wrote his paper on the lifecycles of humble-bees in New Zealand in 1954.

In this paper, Cumber stated that there were three species present in New Zealand but he only made reference to the aforementioned two. His information was sourced from L. J. Dumbleton's 1948 paper in which it is stated that *B. ruderatus* and *B. terrestris* are widely distributed in New Zealand and a third species, *B. subterraneus* has a more restricted range in the South Island. Dumbleton declared that there was no evidence of *B. lucorum* or *B. hortorum* being present. In 1964 however, Lou Gurr conducted an extensive collection at 265 South Island sites and confirmed *B. hortorum* was indeed present.

Lou Gurr took it upon himself to encourage the establishment of *B. hortorum* and *B. subterraneus* in the North Island and in 1965 he facilitated the shipment and release of 100 *B. hortorum* queens, followed by 38 *B. subterraneus* a month later (Gurr, 1972). All queens originated from South Canterbury and each release occurred at Massey University in Palmerston North.

Surveys over the next three years showed that *B. hortorum* had successfully begun nesting and reproducing in the area but the same could not be said for *B. subterraneus*. No *B. subterraneus* have been recorded in the Manawatu, or anywhere else in the North Island, since 1966 (Gurr, 1972).

Current Distribution of Bumblebees in New Zealand

The most extensive survey of the locations each Bumblebee species can be found was carried out by Rod Macfarlane and Lou Gurr from 1975-1991 in the North Island and from 1969-1993 in the South Island. The results were published in 1995 and since then this paper has been used as the standard for describing each species' distribution (Macfarlane & Gurr, 1995).

The method of gathering the data to create the distribution maps means that the North Island distribution information is now at least 23 years old. There is a good chance ranges would now be different.

Of particular interest is the range of the less common species, *Bombus hortorum*. At the time of the survey's publishing, *B. hortorum's* range was said to be limited to the Manawatu and regions south of there. Not a lot of expansion appeared to have happened since Lou Gurr's 1965 introduction. This statistic was quoted as recently as 2010 (Howlett & Donovan, 2010) but there are suspicions that this may now be inaccurate.

Around the Waikato region there have been reports of bumblebees which resemble *B. hortorum* (D. Pattemore, *pers. comm.*, 2013). This region is over 250km north of where the species' range purportedly ends. A claim like this is not easy to confirm because of the cryptic morphology shared by workers of species *B. hortorum* and the more abundant *B. ruderatus* (Prys-Jones & Corbet, 1987). Differentiating between the two has been a conundrum facing researchers for over a century, even more so now that *B. ruderatus* is extremely rare in the United Kingdom and correct identification is imperative (Ellis *et al*, 2005).

Modern genetic techniques are invaluable when working with cryptic species. In 2006 a digestion site was found to be present in the mitochondrial DNA of *B. hortorum,* but not *B. ruderatus* (Ellis *et al*, 2006). This means it is now feasible to confidently identify when a cryptic worker bumblebee belongs to species *hortorum* using only basic molecular biology.

This innovation leads to the first aim of this thesis: to establish the current distribution of *B. hortorum* in the North Island of New Zealand using the digestion site in the cytochrome b region of the mitochondrial DNA sequence.

Bombus Species' Developing Role in New Zealand Agriculture

The benefits of the bumblebees' presence in New Zealand have become much more apparent in the last decade. As mentioned previously, from the late 1800s bumblebees were only specifically utilised for a limited selection of crops and were otherwise largely unmanaged in New Zealand. Since then the number of crops which are acknowledged to benefit from bumblebee pollination has significantly increased. It is now known to be advantageous to have bumblebees present in crops of kiwifruit, blueberry, bok-choy and pear, to name a few (Howlett & Donovan, 2010; Zisovich *et al*, 2012).

Another reason for their increasing popularity is the rise in cost to use honey bee hives for pollination services. Higher prices are the consequence of several changes in New Zealand's apiculture: firstly, the introduction of the Varroa destructor mite in 2000 had a devastating effect on New Zealand's managed and feral honey bee colonies (Goodwin, 2004). The mite's presence led to a general increase in the cost of managing hives as much care must be taken to prevent or combat a Varroa infestation (Howlett & Donovan, 2010). It is also very uncommon to find wild honey bee hives now and that has removed a large portion of free pollination services horticulturists used to benefit from.

Global demand for Manuka honey, and the subsequent large potential for profit by producing it, has also caused the cost of acquiring hives for pollination to leap significantly (MPI, 2013). A large shift in apiarists producing honey rather than providing pollination services means more must be paid in order to bring hives back for pollination. This 'special' honey comes from bees foraging on the flowers of the native tea tree *Leptospermum scoparium* (known locally as Manuka) and is believed to afford medicinal properties when used as a topical ointment for wounds or ingested when ill (Mavric, 2008).

Not only is it often beneficial to deploy bumblebees to supplement the work of honey bees, in some instances they can even exceed their pollination ability (Zisovich, 2012). Their larger size means a greater surface for pollen to be carried on and they are willing to forage in harsher weather conditions than *A. mellifera* (Corbet *et al*, 1993). They will often be the first to begin working and the last to stop on any given day. It is not unheard of to see a bumblebee out in temperatures lower than 10^{o}C but honeybees appear to require temperatures of at least 12^{o}C to begin foraging (Corbet *et al*, 1993).

It has been possible to purchase cardboard boxes containing established *Bombus* nests for pollination in Europe since the late 1980s. These are particularly popular in greenhouses for crops like tomato which bumblebees 'buzz-pollinate' (Asada & Ono, 1996). This special vibrating behaviour induces the release of pollen, saving labour costs for large companies who would have to perform this activity by hand otherwise (Chen & Hsieh, 1996). These boxes can be used for outdoor crops but with more variation in lifespan and more susceptibility to damage from weather or other animals (D. Pattemore, *pers. comm.,* 2013).

It is possible to purchase bumblebee boxes in New Zealand too, and these always contain the nests of *B. terrestris.* The suppliers encourage the use of their bumblebees outdoors and even suggest removing honey bees altogether in some situations ("Bumblebees as Pollinators", 2011). Many horticulturists would be hesitant to go without honeybees though so the use of bumblebees may result in extra costs or little savings. The ideal situation for growers would be to have ample bumblebee nests occurring naturally in the area surrounding their crops so a high quality pollination service is available without the additional costs (Donovan & Wier, 1978).

This leads to a question: how can the number of bumblebees present in orchards or agricultural landscapes be increased to a level that will reduce the need for expensive honey bee or purchased bumblebee pollination? Particularly for those crops that are known to benefit most including blueberry, pear, lucerne and kiwifruit (Howlett & Donovan, 2010).

Domiciles: Potential to Increase Bumblebee Abundance in Crops

One answer to this question is to provide a readymade nesting site, known as a 'domicile', that simply requires a fertilised queen to discover it and begin building cells and laying eggs (Fye & Medler, 1954). Domiciles have been trialled in quite a few studies internationally, different designs and great variations in success have been published but generally low acceptance rates are the norm. For example, Fye and Medler trialled 154 domiciles of four different designs in Wisconsin, USA and had only 34% occupancy across all designs tested. The most successful trial was wooden boxes placed at the base of fence posts with 15 nests being established there. An attempt to replicate the Fye and Medler approach in Southern Alberta, with a slightly altered design, had rates around 10% occupation for boxes placed beside a fencepost in a field of alfalfa (Hobbs *et al*, 1960).

Another study reports on the efficacy of six different domicile designs (Lye *et al*, 2011). The domiciles were placed in four different areas: botanical gardens, suburban gardens and woodland on a University's grounds in Southern England, and also on Scottish farms. Subterranean domiciles placed in the botanical gardens had the highest uptake rate with 9 out of 20 being occupied. Suburban gardens had the worst occupation with no nest establishment apparent. The domiciles in the woodland and Scottish agricultural sites each had very low nest rates. Out of all the domiciles occupied, there was only evidence that two or three nests had belonged to species *B. terrestris*.

New Zealand trials have also found that *Bombus terrestris* appears to be unsatisfied with the nesting conditions found in a domicile (Donovan & Wier, 1978; Barron *et al.*, 2000). In 1971, Donovan placed 105 nest boxes of varying designs around the Lincoln area in the South Island of New Zealand. Only 15 became occupied. The next year Donovan adjusted the domiciles in accordance with the previous year's findings about which design features were preferred. Over the whole three year study, only 9.5% of the total occupancy was attributable to *B. terrestris.* More than 50% of the hives were occupied by *B. hortorum* so something in the design must be appealing to this species

much more than *B. terrestris* and may be indicative of differences in the natural nesting habits of the two species.

Barron also carried out a domicile study in Lincoln in 2000 and saw that for 80 domiciles placed in agricultural landscapes over a four year period, the highest occupation rate was only 21% and again, highest occupancy was not by *B. terrestris* but by *B. hortorum* (Barron *et al.*, 2000).

Because it is the most abundant species nationally (Macfarlane & Gurr, 1995), if domiciles were more predictably attractive to *B. terrestris* it could be rather easy to boost their nest density and improve pollination services. The cost of purchasing and installing domiciles (which can stay in place for years if properly maintained) would be substantially less than purchasing bumblebees each year and could reduce the number of honey bee hives required. The domiciles could be left unattended much of the time with only the base nesting materials being replaced as needed and the entrance kept clear.

Bombus terrestris Nesting Habits in the Wild

When determining how to optimise domicile design and placement to attract *B. terrestris,* a review of where this species' chooses to nest naturally is crucial. It is widely accepted as fact that *B. terrestris* is a subterraneous nester (Fussell & Corbet, 1992), finding small cavities below ground level in which to establish their colony, but is there any evidence of other inclinations to nest in a particular habitat type or under certain conditions?

The greatest source of information on *B. terrestris* nesting habits are three studies on bumblebees conducted in the UK which made use of citizen science to obtain far larger samples than is usually possible for this study subject. Unmanaged bumblebee nests are notoriously difficult to find but with the help of the public, Osborne *et al.* (2008) reported data from 232 nests, Fussell and Corbet (1992) were able to gather data from 432 nests and Lye *et al.* (2012) gathered information on a record 1022 nests.

Osborne *et al.* (2008) reported the results of a field survey conducted in 2004 which requested people take a moment during June or July to observe a patch in their own garden and in a countryside habitat to look for forager traffic leaving or entering a nest. Of the 232 nests found, 116 belonged to queens possessing the *B. terrestris* colour pattern. Because this study was conducted by non-experts, asking for species level identification was not feasible for every instance so it is possible those recorded as *B. terrestris* could have also been species *lucorum, soroeensis* or *magnus*. Across all species, the largest number of nests were found in gardens, along fence lines and in hedges, with the lowest number of nests recorded in open grassland and woodland areas. Gardens with compost heaps were found to be more likely to contain ground nesting bumblebee species.

Specific information about the nest locations of *B. terrestris* is rather hard to infer from the published results. The data indicates nests of bees with *B. terrestris* morphology were somewhat evenly dispersed between areas that contained grasses, stones, woodland features, anthropological features, and 'other' in both Osborne *et al* (2008)

and Fussell and Corbet's (1992) surveys. It appears there may have been a slight preference for nesting in areas with a stone based ground covering in the 2004 survey but whether or not this was statistically significant is not reported. Many of the results of the Osborne *et al* survey had a high level of similarity to the results of the study conducted by Fussell and Corbet from 1989-1991, and both were written in such a way that it is hard to pull out specific information relating to only *B. terrestris*.

Lye *et al.'s* paper published in 2012 has by far the largest number of bumblebee nests ever reported in one study. Citizen science led to data from 1022 nests being amalgamated. 292 of these belonged to *B. terrestris*; in this study the species was confirmed by asking volunteers to send in photos of the bumblebees occupying the nests they found.

The study reports that all species appeared to be generalists in their nest site preferences and there was a high level of overlap between species' nest site locations. 31% of *B. terrestris* nests were found in a 'hole in the ground', this was the highest percentage followed by 20% 'under building/man made structure' (Lye *et al*, 2012). No further information was provided about what was in the immediate vicinity of either the holes in the ground or the anthropological features. This is a large gap in the data which could have provided a lot of extra insight into nest selection.

Kells and Goulson (2003) concluded that there is evidence for interspecific differences in nesting preferences, based on where queens choose to *search* for nesting sites. They observed 1287 instances of nest searching behaviour by seven of the more common bumblebee species in a Southern UK agro-ecosystem. Their study showed *B. terrestris* appeared to prefer searching along banks, some with tussock grasses, but showed no preference between open ground and mixed forest edge habitat types.

Svensson (2000) also found differences between species in preference for landscape type and habitat. In this study of nest searching behaviour the focus for *B. terrestris* appeared to be along field boundaries and open ground patches with an abundance of withered grass and tussock, similar to Kells and Goulson (2003). Queens were far less likely to be encountered in the centre of a field than anywhere else.

When all the information provided in the aforementioned studies is combined, it appears that *Bombus terrestris* queens are most likely to nest in pre-existing holes in banks that are covered with stones and tussock grasses and least likely to be found nesting in the centre of a managed field. The limitations of each study and the tendency to generalise across species make this interpretation difficult to accept though. One would be wise to hesitate before investing time or money based on this claim. This lack of knowledge about what makes an attractive *B. terrestris* nesting site leaves much opportunity for research.

The second aim of this thesis therefore is to investigate the nesting habits of *Bombus terrestris* queens to search for a pattern of preferences which can be applied to domicile design and placement in a New Zealand setting.

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Chapter Two

Revising the distribution of *Bombus hortorum* in New Zealand

Abstract

The presence of two morphologically cryptic bumblebees (*Bombus* spp.) in New Zealand, *B. hortorum* and *B. ruderatus,* makes defining species distributions difficult. This paper aims to establish the current distribution of species *B. hortorum* which has historically been recorded as having the more restricted range of the two species. The discovery of a restriction site in the cytochrome b region of the mitochondrial DNA means delineation is now possible. A *B. hortorum* specimen will result in two bands of DNA on a 3% agarose gel post digestion. A *B. ruderatus* specimen will not digest leaving a single band of DNA visible. A call for cryptic specimens to be posted to the researchers was disseminated across the country. The 227 bumblebees received had DNA extracted for PCR using the HotSHOT protocol and amplifications were digested using the Tsp45I restriction enzyme. The success of this process confirmed that the range of *B. hortorum* extends at least 250km further north than previously reported. The same methodology was applied to 26 cryptic museum specimens ranging in age from 1953-2013. Only eight of these samples had DNA amplified successfully and only four yielded visible bands on a post digest gel. An ambiguous banding pattern was observed comprised of three bands which did not resolve to two bands after exposure to more enzyme. Sequencing of PCR amplicon of the specimens in question revealed two distinct haplotypes were present; one susceptible to digestion and one which lacked the restriction site. Following the low success of the HotSHOT protocol for extracting DNA from museum specimens, the method was tested against a DNeasy kit and a 'Salting Out' protocol on 16 additional museum samples. Results indicate that the HotSHOT protocol, although highly effective on fresh DNA, may be suboptimal for use on museum specimens.

Introduction

A survey of the New Zealand distribution of bumblebees reported that species *Bombus hortorum* was ubiquitous to the east of the Southern Alps in the South Island but extended only so far as the Manawatu region in the North Island (Macfarlane & Gurr, 1995). This species had been released near Palmerston North in the Manawatu in 1965 (Gurr, 1964) and by 1995 appeared to have extended its range south into the Wellington region but it was not to be found in any other North Island regions.

Although the North Island distribution data was collected between 1975 and 1991, the resulting distributions have been considered accurate since their publishing and have been cited as recently as 2010 (Howlett & Donovan, 2010). However, reports from the Waikato region of New Zealand of sightings of bumblebees resembling *B. hortorum* call into question the distribution data (Pattemore, Personal Communication, 2014). Relatively recently introduced species such as *B. hortorum* are likely to continue expanding their range over time, if the environment permits them to do so, and therefore regular surveying is required to maintain accuracy in reporting.

Unfortunately, there is a problem facing those working with this species which prevents easy confirmation of presence or absence: they share external morphology with *B. ruderatus*. Both have a black body with two yellow bands on the thorax, another yellow band on the top of the abdomen and a white 'tail'. Individuals can be differentiated by experts with between 89-100% accuracy (Ellis *et al*, 2006) but for non-experts it is exceedingly difficult, especially when dealing with the worker caste.

Even in highly regarded *Bombus* keys (e.g. Prys-Jones and Corbett 1987), the final couplet does not provide differentiation between *B. hortorum* and *B. ruderatus* and these species are described as difficult or impossible to distinguish. Williams and Hernandez (2000) attempted to identify the most reliable morphological traits for species assignment but could only conclude that breadth of head and length of malar area would allow distinction for most queens, but not all. For workers, specific traits related to their pubescence showed promise of providing separation but no other characters were reliable (Williams & Hernandez, 2000).

In 2006 a breakthrough was made: a simple digest can now be carried out on amplified mitochondrial DNA to assign species to a cryptic specimen (Ellis *et al*, 2006). The development involves the restriction enzyme Tsp45I digesting a site of the mitochondrial cytochrome b gene of *B. hortorum* but not *B. ruderatus*. If gel electrophoresis of digested PCR products, of the cytochrome b gene, reveals two bands then this indicates the DNA is from *B. hortorum*. No digestion, only the original DNA band being visible, is indicative of *B. ruderatus*.

This simple and effective tool is utilised in the present study with the aim to update the distribution of *B. hortorum* in the North Island of New Zealand. If *B. hortorum* is present in the northern regions of the country this will be positive news for growers of red clover; a greater number of long tongue species working alongside the more

abundant short tongue, *B. terrestris*, can provide much more efficient pollination and higher seed yields (Gurr, 1974; Donovan, 2001; Palma *et al*, 2005)

It should also be taken into consideration that, due to the difficulty in identifying the two cryptic species, there is a chance that during the 1995 survey, some bees may have been misidentified. Therefore, another aim of this paper is to review the previously published range of *B. hortorum*. Specimens collected by Macfarlane and Gurr have been kept in storage and if enough DNA can be extracted from them their specific identity can be confirmed using the DNA digest identification tool. Specimens collected north of Palmerston North will be of particular interest as their species assignment could provide evidence to suggest *B. hortorum's* reported range was erroneous in 1995 (Macfarlane & Gurr, 1995).

Methods

Approach

In order to determine the current distribution of the two cryptic bumblebee species in New Zealand, species assignment was undertaken using the mitochondrial cytochrome b gene restriction enzyme protocol (Ellis *et al*, 2006). DNA was extracted from modern bumblebees using the HotSHOT protocol (Truett *et* al, 2000) by geneticist James Sainsbury at the Institute for Plant and Food Research New Zealand (Ruakura). MtDNA Cyt b PCR amplification products were digested with Tsp45I restriction enzyme. Capture locations for each of the cryptic bee species (based on restriction enzyme analysis) were mapped using BatchGeo.com. Approximately 10% were excluded from analyses based on morphological identification as *B. terrestris*.

A similar approach with minor adjustments to DNA restriction conditions was applied to 26 bumblebees from historic samplings at Massey University to determine whether there were misidentified records of *B. hortorum* at locations beyond the recognised range.

The identification of an anomaly in the observed banding pattern of two specimens post digestion lead to DNA sequencing being carried out to try and establish the cause of the unique banding.

Finally, alternate extraction methods were reviewed to assess their suitability for extracting DNA from museum specimens. Three methods were tested on 16 bees of differing preservation ages from Massey University's collection: HotSHOT (Truett *et al*, 2000), DNeasy Kit (Qiagen©) and 'Salting Out'. Three legs were removed from each bee and prepared separately for extraction, thus allowing the quantity of DNA resulting from each method to be compared on a per specimen basis.

Sampling Bees

Fresh Specimens: A citizen science approach was utilised as the most efficient way of obtaining samples of bumblebees displaying the *B. ruderatus/hortorum* cryptic

morphology. A letter was sent out to various individuals, interest groups and organisations throughout New Zealand requesting people look in their garden or neighbourhood for the relevant bees and post to the researchers (see appendix 1 for example letter). 227 specimens were received by the researchers.

Cryptic Museum Specimens: 26 specimens from past surveys, including Macfarlane and Gurr's, which displayed the cryptic morphological traits belonging to *B. ruderatus* and *B. hortorum* were borrowed from the Auckland War Memorial Museum's entomology department, the New Zealand Arthropod Collection and Massey University's Entomology department. Initial preservation methods were unknown but all were dried and pinned in storage. Specimens were from: 1947 (x4), 1953 (x3), 1954, 1968, 1981 (x3), 1982 (x3), 1983 (x3), 1991 (x4) and 2009 (x2) 2010 and 2013.

Extraction Method Test Specimens: 16 specimens were selected from the Massey University entomology collection to provide dried tissue samples for comparing three extraction methods. Initial preservation methods were unknown but all bees were dried and pinned in storage. As this experiment was only testing the DNA extraction methods, the species of the bees was not important. Specimens were from: 1972 (x2), 1978 (x2), 1993 (x2), 1997 (x2), 1998 (x2), 1999 (x3), 2001 and 2004 (x2).

Sample Preparation

Each *Bombus* spp tissue sample was prepared in the same way. A leg was removed from the specimen, placed in a 1.75mL micro centrifuge tube and then cut up finely to maximise surface area exposed to tissue lysing agents. Post extraction samples were frozen at -20^{o}C.

DNA Extraction Methods

HotSHOT: This procedure involves placing a tissue sample in 75µL of hot alkaline lysis reagent containing 25 mM NaOH and 0.2 mM disodium EDTA (pH 12) then incubating for 10 minutes at 95^{o}C. This is followed by neutralisation in 75µL of 40 mM Tris-HCl (pH 5). The method was developed as a quick and easy way to prepare tissue samples from laboratory mice for PCR (Truett *et al*, 2000).

DNeasy Genomic DNA Isolation Kit (Blood and Tissue, Qiagen©): For this protocol, 180µL of ATL buffer was added to each prepared leg in a 1.75mL tube. 40µL of proteinase-k was added, mixed by vortex, then the samples were put in a moving incubator for 5 hours at 55^{o}C.

When samples were removed from the incubator, they were vortexed gently for 15 seconds then 200µL of buffer AL was added. This was shaken and pipetted up and down to mix. Samples were put on a hotplate at 70^{o}C for 10 minutes then 200µL of 100% ethanol was added and mixed.

Collection tubes (2mL) with columns inside were assembled and approximately 500µL was pipetted onto each column from each 1.75mL tube containing solution. These were spun at 8000rpm for one minute then the collected liquid and 2mL tubes were discarded. The columns were placed inside another set of 2mL collection tubes and

500ml of wash AW1 added to each. These were spun at 8000rpm for one minute then liquid and tubes discarded again. 500µL of AW2 was then added to each column in new 2mL collection tubes and spun for three minutes at 13000rpm. The collection tubes and liquid were discarded for a final time.

The columns were placed in 1.75mL micro-centrifuge tubes and 50µL of buffer AE added. These were incubated at room temperature for one minute then spun at 8000 for one minute. This last step was repeated for each column resulting in a final elution volume of 100µL.

Salting Out: DNA extraction with the "salting out" protocol followed the same methods as Sunnuck and Hales (1996). Prepared legs were combined with 7.5µL of proteinase K solution (10mg/µL) and 300µL of TNES buffer (comprised of TRIS 1M, NaCl 5M, EDTA 500nM, 5% SDS). The sample was incubated at 37^{o}C for approximately 18 hours.

When samples were removed from the incubator, 85µL of NaCl (5M) was added. Samples were shaken well to mix. The solution was spun at 13000rpm for 5 minutes and then 350µL of supernatant was pipetted off into a new 1.75ml micro-centrifuge tube and 400µL of ice cold 100% ethanol was added. This was spun at 13000rpm for 30 minutes.

Following this, all ethanol was poured off and 400µL of 70% ethanol was added as a rinse. This was mixed by inversion, spun at 13000rpm for 5mins then poured off. Samples were left to air dry on a warm hotplate to remove remaining alcohol. DNA was then re-suspended in 50µL of H_2O, mixed by inversion and gently vortexed to the bottom of the tube.

For all extraction methods, a negative sample with no organic matter was carried through from start to finish to check for contamination.

PCR and Endonuclease Digestion

A fragment of the cytochrome b gene was amplified from each sample via the polymerase chain reaction (PCR) using the iNtRON Biotechnologies Maxime PCR pre-mix (i-taq$^{(Life\ Technologies™)}$). Primers were designed to amplify a 5bp fragment bridging a point mutation characteristic of the haplotype found in *B. hortorum*.

Forward primer: TTCAGCAATTCCATATATTGGAC.

Reverse primer: ATTACACCTCCTCATTTATTAGG.

Each pre-mix PCR tube contained 0.65µL forward primer, 0.65µL reverse primer (both at 6.5 pmol), 2µL of DNA extract and 16.7µL of milli-q water. Tubes were placed in the PCR machine and run on the following regimen: 94°C for 2 min followed by 35 cycles at 94°C for 20 seconds, 48°C for 10 seconds and 72°C for 20 seconds; with a final 2 min extension period at 72°C then cooled to 10^{o}C. 3µL of each PCR product was run on a 1% agarose gel at 110V for 28 minutes to check for amplified DNA.

DNA was prepared for digestion in tubes containing 3-4.5µL of PCR product, 0.2-0.4µL of the restriction enzyme Tsp45I (New England Biolabs) and 1 x cutsmart buffer (New England Biolabs).

The digestion was carried out at 65ºC for 2-5 hours and the products were run on 3% agarose gels at 60V for 180 minutes (*Figure 2.1*). Conditions were varied to ensure maximum enzymatic digestion.

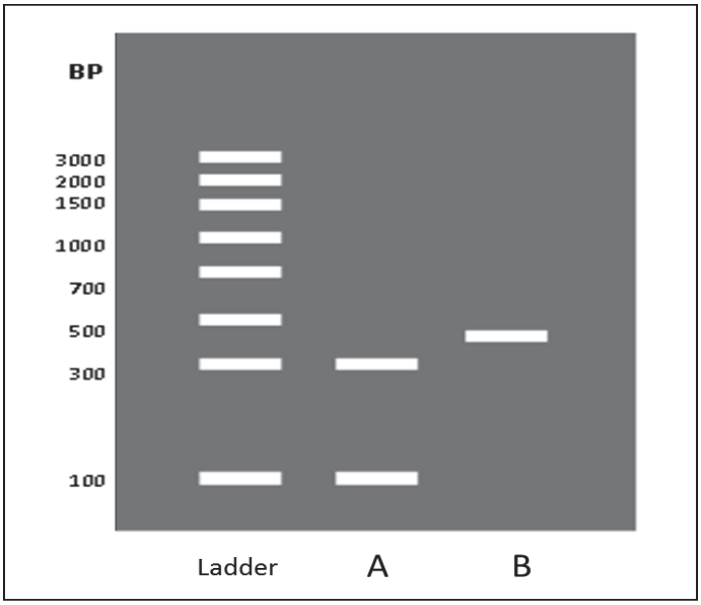

Figure 2.1: Expected post-digest DNA banding patterns showing two bands indicative of B. hortorum (A) resulting from a cut at the Tsp45I restriction site. A single undigested band is present for samples of B. ruderatus (B) which lack the restriction site (Ellis et al, 2006).

NanoDrop

The DNA content of each extraction was quantified using the NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific). Also recorded were the absorbance ratios at 260/280nm and 260/230nm which can provide an indication of DNA 'purity'. A 260/280nm ratio lower than 1.8 is indicative of contamination by protein in the sample. An ideal 260/230 ratio would be near 2.0. Lower than 1.8 suggests the sample is contaminated by organic compounds (Glasel, 1995).

DNA Sequencing

DNA sequences of four specimens, two showing a unique three band pattern post digestion and two which had their specific identity confirmed, were determined using BigDye® Terminator v3.1 chemistry (Applied Biosystems™) and 3730 DNA analyser capillary(Applied Biosystems™) to try and establish the cause of the unique banding pattern.

Data Analysis

All analysis of data was carried out using R statistical software version 3.1.2 (2014) unless stated otherwise.

Results

Cryptic Species Assignment – Modern Specimens

A total of 227 specimens that displayed the *B. ruderatus/B. hortorum* cryptic morphology were received and processed at The Institute for Plant and Food Research in Ruakura. Forty six were identified as *B. hortorum* post digestion and six of these were from locations north of existing records (*Figure 2.2)*.

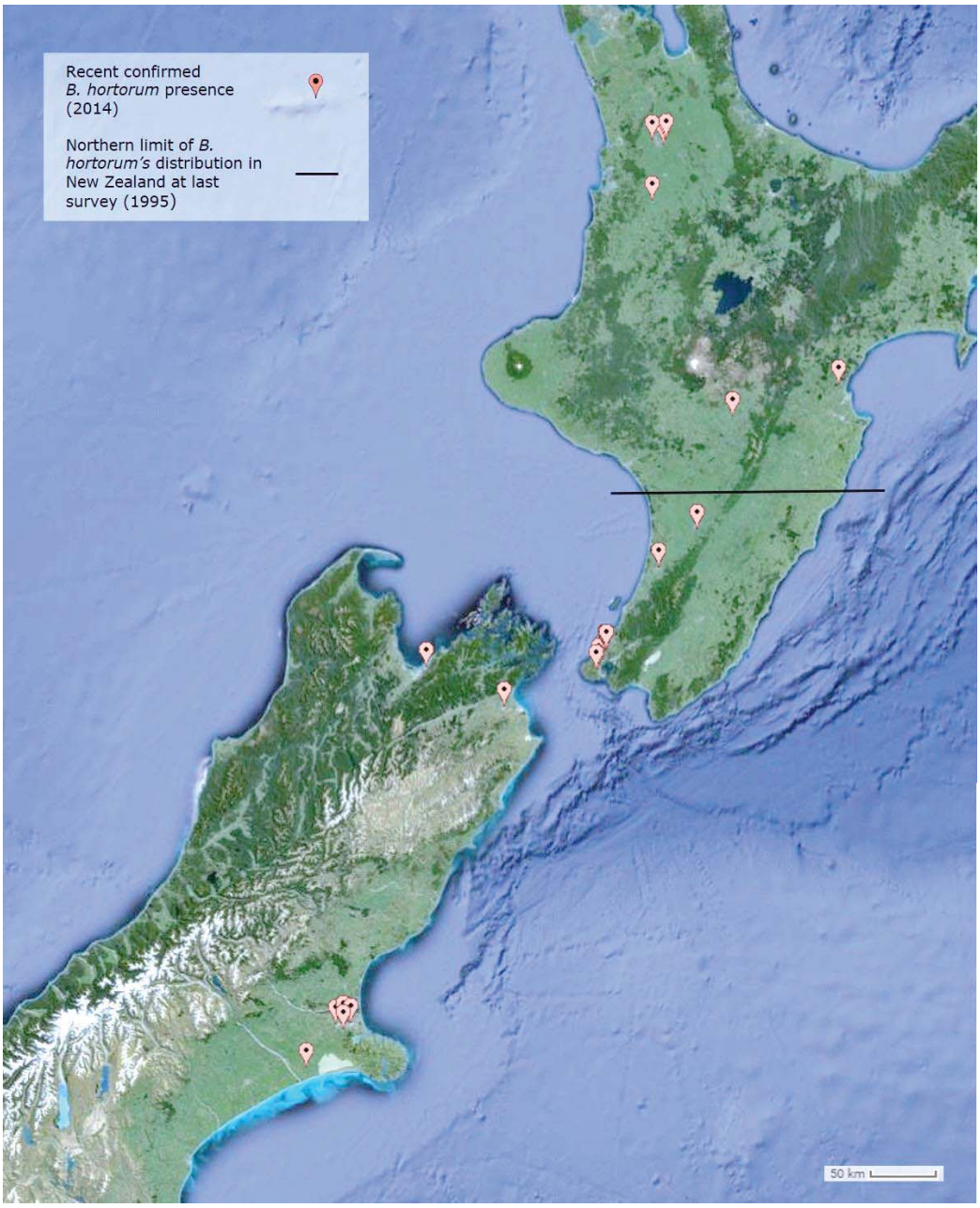

Figure 2.2: Map of New Zealand showing the catch locations of confirmed specimens of B. hortorum in this study. Black horizontal line indicates previously recorded northern limit of B. hortorum, as identified by Macfarlane and Gurr (1995). An additional point is included at Otorohanga in the Waikato region where a specimen from 2013 was collected that has been confirmed as B. hortorum in the present study. Map created using BatchGeo.com.

Cryptic Species Assignment - Museum Specimens

DNA extraction using the HotSHOT protocol yielded PCR products in eight of the 26 specimens that ranged in age and origin (*Table 1*). Unfortunately the success rate was very low and only eight specimens were able to have amplified DNA digested with the Tsp45I restriction enzyme (*Figure 2.3)*.

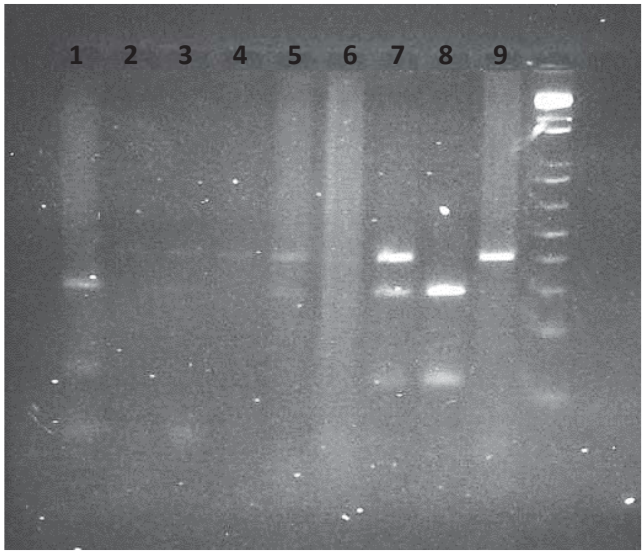

Figure 2.3: Image of 3% agarose gel showing the banding patterns of post PCR DNA that has been exposed to the restriction enzyme Tsp45I for 5 hours at 65ºC.

Three DNA banding patterns were observed (*Figure 2.3*). A single undigested band (well 9) indicated the absence of a digestion site, in this case because the specimen was *B. terrestris*. Two bands of DNA in wells 1 and 8 indicated a successful digestion and allowed species assignment as *B. hortorum*. An unusual digestion product of three bands (wells 5 and 7) was observed. This can be indicative of contamination or a partial digestion but it remained even when the process was repeated with extra enzyme, ruling out partial digestion as a cause.

Table 2.1: Museum specimens' species assignment after digestion with 0.4µL of restriction enzyme Tsp45I (New England Biolabs). A confirmed B. terrestris was included in the digestion as a negative control. A dash (-) represents an ability to detect any bands of DNA on the gel. 'Indeterminable' describes those specimens which digested to reveal three bands of DNA.

Well ID	***Specimen ID***	***Year***	***Collection Location***	***Region***	***Species***
1	054	1953	'Brook Street'	Canterbury	*B. hortorum*
2	72a	1972	Mount Peel Station	Canterbury	-
3	72b	1972	Mount Peel Station	Canterbury	-
4	78a	1978	Tekapo	Canterbury	-
5	AM045	2009	Tupane Lake, South Kaipara	Auckland	*Indeterminable*
6	AM046	2009	Tupane Lake, South Kaipara	Auckland	-
7	2010	2010	Levin	Manawatu	*Indeterminable*
8	2013	2013	Otorohanga	Waikato	*B. hortorum*
9	04b	2004	Waikanae	Wellington	*B. terrestris*

Unique Banding Pattern: DNA Sequencing

The unexpected presence of three DNA bands during gel electrophoresis, even after repeated digestion, suggests a mixture of sequence variants. To examine this possibility, cytochrome b PCR products for the bees of interest and a control were sequenced. The resulting chromatograms confirmed that the bees with DNA yielding three banded digestion results were ambiguous at the restriction enzyme recognition site (*Figure 2.4*).

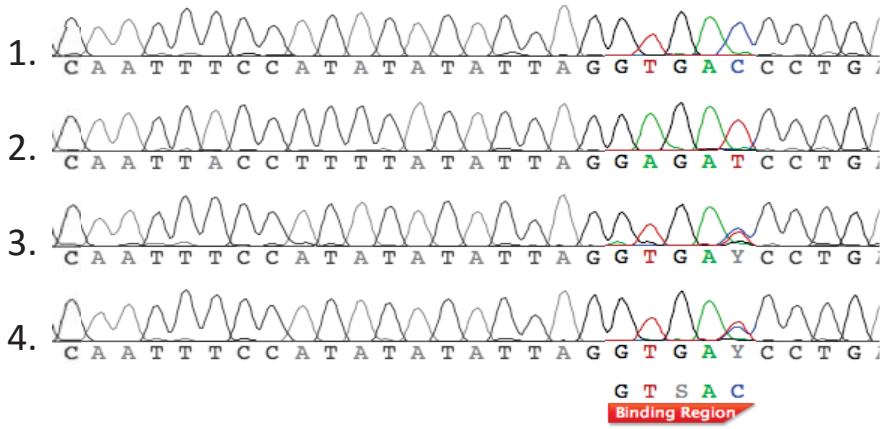

Figure 2.4: Segment of chromatograms from sequences of cytochrome b gene PCR amplified from DNA of four bumblebees (1. 2013 (B. hortorum), 2. 2004 (B. terrestris), 3. 2009 (indeterminable), 4. 2010 (indeterminable)). The restriction recognition site of the Tsp45I endonuclease enzyme is indicated with 3' site on the ambiguous region. Hence, top sequence is full restricted (cutting of PCR product), while second sequence was not cut. Sequences 3 and 4 resulted in partial digests because two different sequences (haplotypes) are present.

Comparison of Three Extraction Methods

PCR was carried out on all samples then 2µL of each resulting solution was subjected to gel electrophoresis check for amplification (*Figure 2.5*).

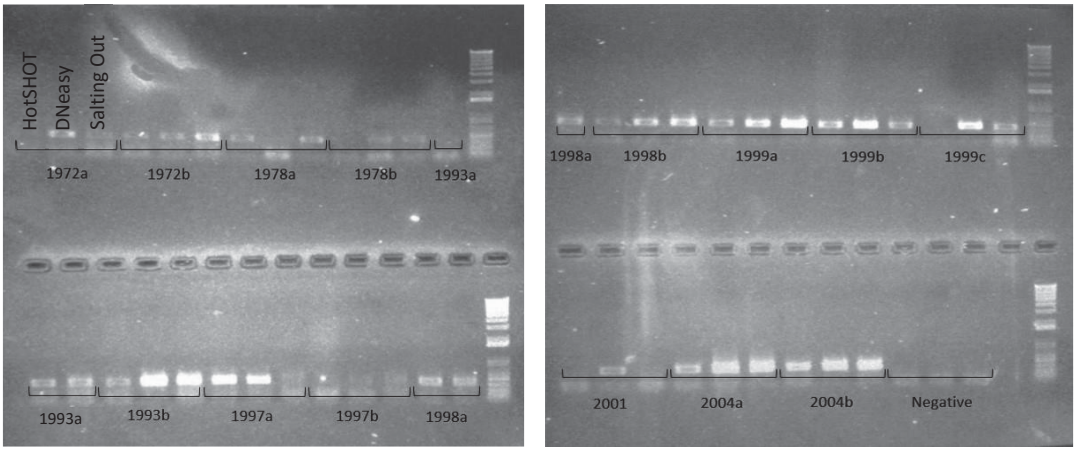

Figure 2.5: Images of 1% agarose gels showing PCR products of three separate extraction techniques (hotSHOT, DNeasy kit and Salting out) trialled on 16 dried bumblebee samples ranging in age from 1972 to 2004.

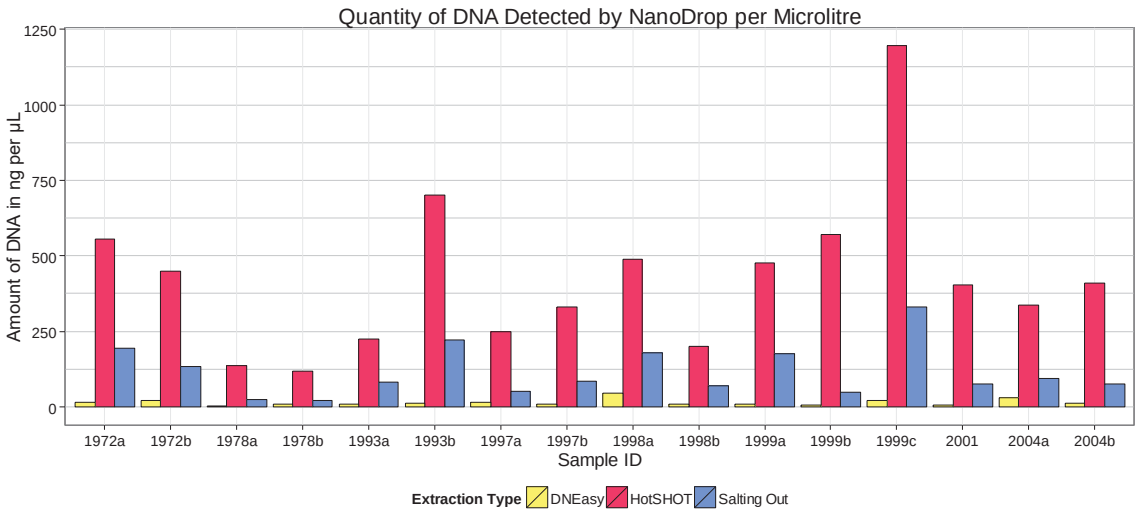

Figure 2.6: DNA concentration in (Ng/µL) in the stock extraction solution for each of three different extraction methods: DNeasy (yellow), HotSHOT (red) and Salting Out (blue). Measurements were taken using the NanoDrop 1000. All three methods were trialled on each specimen.

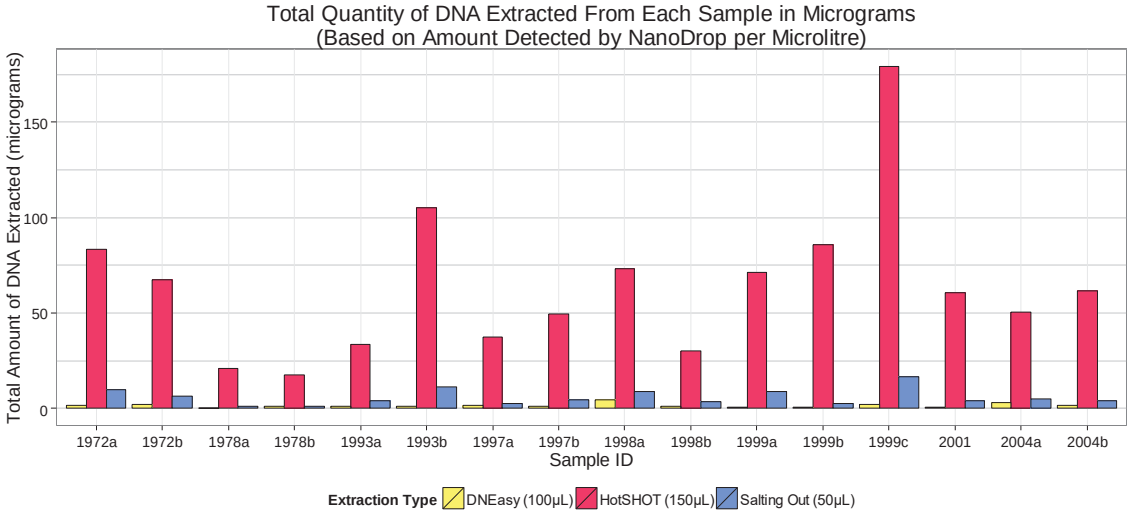

Figure 2.7: Comparing total DNA yield per leg of bumblebee using three extraction methods: DNeasy (yellow), HotSHOT (red) and Salting Out (blue). Total yield = concentration (Ng/µL) x total extraction volume (µL).

NanoDrop readings were taken for every stock extraction to get an indication of how much DNA each method yielded (*Figures 2.6 & 2.7*).The HotSHOT protocol had the highest readings for quantity of DNA per microlitre for every specimen (mean 427.8 Ng/μL) but the average 260/280nm and 260/230nm ratio values were the furthest from ideal at 1.548 and 0.739 (*Table 2*).

Table 2.2: Average readings taken from the NanoDrop 1000 for each extraction method trialled on 16 samples.

Extraction Method	***DNA Quantity (mean)***	***260/280 mean***	***SD***	***260/230 mean***	***SD***
HotSHOT	427.8 Ng/μL	1.548	0.220	0.739	0.088
DNEasy	14.5 Ng/μL	2.04	0.344	1.133	0.649
Salting Out	116.2 Ng/μL	1.833	0.149	1.005	0.258

The average 260/280nm and 260/230nm ratios were compared for each method and significant differences were found ($p < 0.05$) between the HotSHOT and DNeasy and the HotSHOT and Salting Out protocols (*Table 3*). This may be indicative of the nature of the extraction method, suggesting chemical and organic contamination is left suspended in the solution when using the HotSHOT method. There was no significant difference found between the DNeasy and Salting Out protocols and the 260/280nm, 260/230nm ratios were closer to ideal for these methods.

Table 2.3: Results of comparison of means testing carried out using the average 260/280nm and 260/230nm absorbance ratios recorded in table 2.

t-Tests 260/280	***t-statistic***	***Degrees of freedom***	***p-value***	***Significant at 0.05?***
HotSHOT & DNeasy:	4.82	25	**= 0.00002**	Yes
HotSHOT & Salting Out:	4.29	25	**= 0.00012**	Yes
DNeasy & Salting Out:	2.209	19.9	= 0.97952	No
t-Tests 260/230	***t-statistic***	***Degrees of freedom***	***p-value***	***Significant at 0.05?***
HotSHOT & DNeasy:	2.406	15.1	**= 0.01333**	Yes
HotSHOT & Salting Out:	3.903	17.9	**= 0.00058**	Yes
DNeasy & Salting Out:	0.733	19.1	= 0.76607	No

Discussion

Cryptic Species Assignment

The use of citizen science to gather modern specimens was highly successful. It was expected that some *B. terrestris* bees would be sent in despite the information provided to the public but the vast majority received were the correct putative species providing a good sample size overall.

Sampling was however bias towards the Canterbury region of the South Island and Waikato in the North Island. It would have been useful to have more specimens from regions like Taranaki, Bay of Plenty and the Central Plateau to build a more precise distribution map north of the previously recorded limit of *B. hortorum's* range.

Five specimens from Waikato did provide positive *B. hortorum* location data confirming the species' range spreads at least 250km further North than existing data suggests (Macfarlane & Gurr, 1995).

The HotSHOT DNA extraction protocol (Truett *et* al, 2000) was effective for obtaining DNA for PCR from fresh samples with a success rate close to 90% using only a single leg from each bee. A larger initial tissue sample and more time spent in the hot lysis buffer could have increased the success rate but as there were a large number of samples to process time was not spent investigating this.

On the basis of the initial success in obtaining amplified DNA via PCR, the HotSHOT protocol was applied to museum specimens ranging in age from 1947-2013. Of the 26 specimens processed only eight were carried through to the restriction enzyme stage and the results of only four of these were visible post digestion: 1953, 2009, 2010 and 2013.

The specimen collected in 2013 from the Waikato region was confirmed as *B. hortorum* but because the date is so recent conclusions cannot be drawn about when the species' range may have expanded, or if the originally published range was incorrect.

Each bee was processed in a non-destructive manner with only a single leg removed to maintain the sample's value in its respective collection. There may have been more success if a larger portion of each bee was able to be used as it is likely the DNA in each leg had degraded significantly. This is especially true for samples over 50 years old, although one of the best extractions came from a bee dated 1953. As with the fresh specimens, more time spent in the lysis stage could also have increased DNA yield.

The results of subsequent analyses to investigate alternate extractions methods suggest the HotSHOT method may be suboptimal at extracting DNA from preserved specimens and that other extraction methods can be more proficient.

The occurrence of an unexpected banding pattern on the post-digest gels of some museum specimens suggest incomplete digestion of the PCR products. Similar instances of this triple-band pattern have previously been recorded as incomplete digestion of *B. hortorum* (Sainsbury, *pers. comm.,* 2014) but even after prolonged digestion time a two band digestion product did not result in some cases.

To investigate this further, mtDNA cyt b PCR products from examples of partial cutting and full or no cutting were sequenced. Alignment of these sequences confirmed that this is not a case of incomplete digestion due to limited enzyme activity (*Figure 2.4*). In these cases, partial digestion was the result of the presence of two different sequence motifs; one with the restriction recognition site and one without. Further research is needed to confirm the source of the two sequences.

Comparison of Three Extraction Methods

The HotSHOT method (Truett *et* al, 2000) appeared to yield the most DNA from museum specimens but these extractions also had the highest amount of contamination, revealed by their low 260/280nm and 260/230nm absorbance ratios. This was to be expected given that everything initially put in to the extraction remains in the final product including any hair or fragments of the bees' legs. Any compounds or organic material could confound the NanoDrop spectrophotometry or inhibit the PCR process which may explain observed discrepancies between the amount of DNA extracted and the PCR products.

The proclivity of the HotSHOT method to shear DNA (Truett *et al*, 2000) may be another explanation for low quality PCR products from museum specimens. The aged DNA is likely to have degraded to shortened fragments and further cutting when subjected to the hot lysis buffer may render most of the extraction unusable, despite a high concentration reading on NanoDrop spectrophotometry.

The significant difference in mean 260/280nm and 260/230nm absorbance ratios between the HotSHOT protocol and the DNeasy Kit (Qiagen©) and 'Salting Out' methods indicates that extracting DNA using these procedures does not result in as much contamination. This may mean less PCR inhibiting factors allowing a stronger amplification providing more copies of the sequence of interest for analyses.

Extraction quality and quantity has the potential to be improved by spending more time altering the specific procedures for each extraction. For example, submerging tissue samples for a longer time in a larger amount of lysis buffer may increase initial DNA yield for the HotSHOT protocol. Experimenting with different dilutions of stock extraction and how this impacts on PCR product may also generate better results across all extraction methods. Time constraints prevented these options being looked in to in the present study as well as the limitations imposed by processing museum specimens in a non-destructive manner.

Despite the technical issues encountered, the aim of the present study was fulfilled; the presence of *B. hortorum* in the Waikato region, north of Palmerston North, has been verified. New location data for Napier and Taihape were confirmed also (*Figure 2.*2). A potential problem with using the Tsp45I restriction site in the mtDNA cyt b gene has been identified though which may limit the accuracy of this site in delineating species. In the present study, the two museum specimen PCR products exposed to the restriction enzyme that displayed an ambiguous three-band pattern could not have a species assigned. Sequencing revealed the presence of two distinct haplotypes (*Figure 2.4*).

Occurrences when species assignment is not possible could be particularly problematic for *B. ruderatus* conservation efforts in the UK, which was the main objective behind the tool's development (Ellis *et al*, 2006). This could be a unique situation within New Zealand populations and pose no problem to species identification and conservation in Europe but caution should be taken. Further research in to the source of the different haplotypes is recommended.

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Chapter Three

Investigating the Nesting Behaviour of *Bombus terrestris*

In New Zealand and the Netherlands

Abstract

Increasing local populations of the most abundant bumblebee species in New Zealand, *Bombus terrestris,* can confer large benefits to those growing crops in which *Bombus* species are highly efficient pollinators. One way to increase bumblebee numbers is by providing queens with artificial nesting sites, known as domiciles, but previous research has shown domiciles to have notoriously low establishment rates, particularly for *B. terrestris*. Knowing what attracts queens to natural nesting sites could allow alterations to the design and placement of domiciles, leading to them being selected preferentially by queens. In the present study research was conducted in large field sites in both the Netherlands (NL) and New Zealand (NZ) to observe nest search behaviour by queens emerging from hibernation. Transects dictated by random sets of coordinates were walked across both field sites for four weeks and information gathered on every nest searching queen encountered. The number of queens seen varied greatly between field sites: 57 in NL and 12 in NZ. It was concluded that queens had a preference for searching under mixed forest (NL, $p < 0.05$) and evergreen forest (NZ), and were drawn to areas with a moss and leaf litter ground cover (NL). Trialling domicile placement under windbreaks or in forests neighbouring orchards and target crops is recommended but further research to increase knowledge about nesting behaviour in NZ specifically may be necessary.

Introduction

New Zealand is home to four species of bumblebee (*Bombus* spp.), all of which were introduced from England in the late 1800s (Gurr, 1957). The most widespread species is *Bombus terrestris* which is found nationwide. *B. ruderatus* is also found throughout the country but is much less abundant than *B. terrestris*. *B. hortorum* and *B. subterraneus* have more restricted ranges with the latter being found only in the Canterbury region of the South Island (Macfarlane & Gurr, 1995).

Bumblebees were originally introduced to the South Island of New Zealand to pollinate red clover, *Trifolium pratense L.* (Dumbleton, 1948). This was instigated when European settlers found that the honey bee (*Apis mellifera*) and New Zealand's native bees were not generating sufficient seed set in this important forage crop. New seeds had to be sown each year to ensure the crop's success, incurring a large cost to farmers (Gurr, 1974).

The long corolla length of red clover flowers requires a heavy insect to push open the petals to reach the pollen and nectar, simultaneously passively depositing pollen that has collected on their bodies, facilitating fertilisation (Gurr, 1974). Smaller insects will rob the flower of its nectar by biting a hole in its base, bypassing the reproductive organs entirely (Fussell, 1992).

Three of the bumblebee species present in New Zealand are considered 'long tongued' which makes them ideal pollinators of clover. Their proboscises range in length from an average of 13.7mm for *B. subterraneus* to 16.6mm for *B. hortorum*. *B. ruderatus* falls in between at 14.5mm (Gurr, 1974). It has been estimated that the pollination service provided by each colony of *B. hortorum* in a red clover seed crop can amount to $1000 worth of seed for the grower (Donovan, 2001).

Unfortunately, the most abundant species, *B. terrestris*, does not possess a long tongue (the average length being 10.2mm) so the workers are inclined to rob red clover nectar like smaller insects would (Fussell, 1992). Large queens can reach the nectaries however and when any caste is specifically collecting pollen they are likely to inadvertently perform some cross pollination. These behaviours, and the relative abundance of *B. terrestris* compared to the other species, mean that despite its short tongue it is still a welcome visitor to this crop (Gurr, 1974).

Bumblebees are now understood to increase the quality of crop pollination in many commercial crops, not just red clover, and can even outperform *A. mellifera* in situations where it is the long standing preferred pollinator (Howlett & Donovan, 2010; Button & Elle, 2014). For instance, it has been observed that by adding *B. terrestris* colonies alongside honeybees in orchards growing the 'Spadona' and 'Coscia' cultivars of pear, average seed set per fruit can be doubled (Zisovich, 2012). Number of seeds is positively correlated with fruit size so the overall quality and yield of the crop will increase compared to when honeybees are the sole managed pollinators.

Studies in kiwifruit orchards also indicate an advantage to having bumblebees present. They show fidelity to kiwifruit flowers when other plants are in bloom in the same area, they spend a short time on each flower so can visit a larger number in total per day and they are able to 'buzz-pollinate' flowers inducing the release of a greater amount of pollen (Read *et al,* 1989; Pomeroy & Fisher, 2002; Minarro & Twizell, 2015).

For these crops and more the willingness of bumblebees to forage in weather that is limiting to honey bees confers an advantage (Corbet *et al*, 1993), even if their pollination ability does not exceed that of honey bees. Bumblebees can be used as an insurance that pollination will continue in bad weather which is important in crops with short flowering times, like kiwifruit.

One of the most promising ways of increasing bumblebee density is by providing secure nesting places for queens in the form of artificial domiciles (Donovan & Wier, 1978). Man-made nesting boxes, commonly constructed from wood with a hole or tube for bees to enter through, are placed in close proximity to the crop requiring pollination before fertilised queens emerge in the spring. The idea is that a nest searching queen will discover the domicile, accept it as her nesting place and establish her colony in a more controlled environment than entirely natural hives. The nest can be monitored and treated for disease as well as being protected from the elements allowing it to grow to a substantial size. At the end of summer it is hoped new queens that are reared will hibernate close by and continue the cycle the following season.

Domiciles have been trialled in different countries where different species reside with large variations in success (Hobbs, 1967; Holm, 1966; Manino *et al*, 2006). In the USA, four different designs were tested across 154 nest boxes with only 34% acceptance rate (Fye & Medler, 1954). In the UK, one study saw six designs tried in four different habitat types with the highest occupation rate being 45% but also one instance of no nest establishment at all (Lye *et al*, 2011).

Similar results have also been observed in New Zealand; Donovan and Barron both conducted studies in the Canterbury region and both had low uptake rates, 15 and 21% respectively (Donovan & Wier, 1978; Barron *et al*, 2000). Most of the nests that were founded belonged to *B. hortorum*, despite *B. terrestris* being the most widespread species. Why it is so reluctant to accept domiciles is not known. It is important to boost numbers of the less common species nationwide but pollination services would likely benefit most if domiciles were selected preferentially by *B. terrestris* due to their relative abundance.

The logical way to increase domicile acceptance is to consider where queens of the species prefer to nest in the wild and then apply that knowledge to domicile design and placement. Previous studies have investigated *Bombus* spp. nesting behaviour, but they did not look at *B. terrestris* exclusively (Fussell & Corbet, 1992; Harder, 1986; Kells & Goulson, 2003). There is evidence for interspecific differences in nest choice, so these studies, which tend to generalise across species or colour groupings, provide little insight into where a *B. terrestris* queen will nest in New Zealand.

Information of this type is difficult to gather; in comparison to honey bee hives, which can contain over 50,000 workers (Farrar, 1968), bumblebee nests are small and often well concealed at ground level (Kells & Goulson, 2003). Forager traffic can be infrequent meaning nest entrances go unnoticed.

The most successful method of gathering information about nesting habits so far has been citizen science. Members of the public in the UK have been asked to send in information about bumblebee nests on three occasions and this has resulted in data from 1686 nests being recorded and studied (Fussell & Corbet, 1992; Osborne *et al*, 2008; Lye *et al*, 2012). Other methods include transect walks to look for nest entrances (Sladen, 1989), transect walks to observe nest searching queens (Svensson *et al*, 2000) and even dogs trained to sniff out nests (Waters *et al*, 2011). As mentioned though, these studies do not go into great detail about the habits of each species individually.

The present study aims to observe and analyse *B. terrestris* queen nest search behaviour using random transect walks. A pattern of characteristics indicating where nests are most likely to be established is the desired outcome. This information can then be applied to domicile design and placement in New Zealand to try and increase acceptance rates. This should have the flow on effect of greater nest density in areas that will benefit from bumblebee pollination services.

Methods

Field Sites

Two sites were used for this research, one in the Netherlands and one in New Zealand (*Figures 3.1 & 3.2*). Having a site in each hemisphere meant that two spring seasons could be studied in the same year. Research was conducted in the Netherlands in March and April and in New Zealand in October and November 2014.

Netherlands

This field site was composed of the adjacent Bussumerheide and Westerheide moors (with a combined area of approximately 4.7 km^2, located at 52.254098N, 5.189581E) that are a part of the larger 27 km^2 Goois Natuurreservaat (Goois Nature Reserve) in central Netherlands, east of Amsterdam between the towns of Naarden-Bussum and Hilversum. The area is owned and maintained by the Gooisch Nature Foundation which aims to preserve the nature and values of the countryside for future generations.

A little over 50% of the land cover for these sites is heathland but there are also prominent forest blocks and grass areas. The heterogeneity of the land cover and variation in topography made this area an ideal field site for assessing habitat selection by *B. terrestris* queens as there are a variety of different habitat types available to the queens during nest searching.

New Zealand

The selected field site was a farm north of Palmerston North, with an area of approximately 3.6km^2 (-40.288876S, 175.647912E). The property was originally used as a dairy farm but is now primarily a grass cropping and beef finishing endeavour. Almost 90% of the land cover for this site is open grass fields but there is also a mix of different blocks including pine, native and fruiting trees. The farm also has variation in topography with several gullies running through which provide good differences in height and slope for the study

Figures 3.1 & 3.2: aerial imagery with blue lines showing the boundaries of the study sites in the Netherlands (left) and New Zealand (right). Images from Google Earth.

Habitat Mapping

The ArcGIS programme ArcMap (v 10.2.2) was used to create a habitat map of each field site where every section of land was classified and colour coordinated based on the type of land use. This included major categories like open field or heathland as well as smaller patches like sand or residential garden.

The classifications were achieved using recent aerial imagery of each area, provided by local councils, and expert knowledge on each site provided by landowners, residents and rangers. Comparisons were also drawn between new and older images on Google Earth when needed as deciduous trees can be hard to discern from images taken during winter months.

Random Points

Over the extent of the land which was designated as the study area for both sites, 200 coordinates were randomly plotted using ArcGIS. From this, subsets of points were selected which were used to conduct the vegetation surveys across each site and also served as start and end points of transects along which the random walks were carried out.

Vegetation Survey

To assess what proportion of different habitats were available to the queens in each area, vegetation surveys were carried out. This involved selecting 110 random points, from the 200 coordinates already created for both New Zealand and the Netherlands, and visiting each to record key information.

The points were loaded onto a handheld Garmin 62s GPS device which would signal when the predetermined coordinates had been reached. When this occurred, a $1m^2$ frame constructed using lengths of plastic tubing was laid on the ground.

From within this frame, information was collected about the different levels of ground cover. This was done by estimating what percentage of the frame each ground cover type filled. For example, a $1m^2$ plot underneath a large oak tree may have a ground cover that is 80% leaf litter and 20% grass whereas a plot out in the open may have moss covering 50% of the soil but also 50% bare ground showing. Difficulty was encountered in the Netherlands heathland where the heath plants often formed a 'canopy' over the ground. Because of this it was deemed appropriate to measure the ground cover in a three dimensional space where the ground may be 100% moss but also covered by a layer of heath which filled approximately 40% of the frame.

The angle of any slope in the ground and the aspect of these slopes was measured using the compass app on Apple iPhone5. At the same time, the top layer of ground was assessed and assigned a soil type.

The distance to the nearest tree from each $1m^2$ plot was also recorded in the field. In most instances it was impractical to take an accurate measurement so an estimate was made which was later checked against the habitat map.

Transect/Queen Encounter walks

Transect walks were carried out across both field sites with start and end destinations determined using randomised numbers (from www.random.org) to generate pairings of points from the initial list of 200 random coordinates. This method of generating transects meant no two transects were the same length or covered the same path, removing the possibility of bias which can be included in human determined transects.

Transects were walked on days where the temperature was expected to exceed 12^oC and when wind speeds were low. The researcher walked at a constant pace from one point to the next while scanning the area in front and to the side for *B. terrestris* queens. When a queen was spotted exhibiting the classic zigzag nest search behaviour or investigating a point on the ground she would be visually tracked until the search behaviour ended (i.e. she flew away or began foraging) or once ten minutes had elapsed. The researcher would maintain a distance of at least three meters from the queens when possible to minimise any potential impact on behaviour.

Coloured flags were used to record information while the queen was still active. Blue flags were used to mark the boundaries of her search area and a yellow flag was placed at any point where she landed and investigated a hole in the ground or plant matter.

Data Collection at Nest Sites

When the observation period had ended, all information about the queen's behaviour was recorded as well as data relating to the location and surroundings for example slope of the ground, habitat type, ground cover, etc. (see appendix 2 for full list of parameters included). The same $1m^2$ frame that was used in the vegetation survey was placed around any holes or points on the ground that were investigated by queens and the same set of data was recorded as in the surveys.

Data Analysis

Data analysis was carried out using R Statistical Software (version 3.1.2, 2014). Data visualisations were generated with the GGPlot2 package. The area of each habitat classification type was determined using the GIS software ArcMap (version 10.2.2). This information was applied to a chi^2 analysis to compare how the queens would have been distributed between each habitat type if their nest site selection was completely random (the expected variable), to where they were actually observed.

Results

Figures 3.3 and 3.4 display the habitat maps generated to assess the proportion of each land use type at the Netherlands and New Zealand field sites. For each site the largest percentage of the land was not covered by forest. In the Netherlands the most common ground cover was heathland (51%). In New Zealand the dominant type was open farmland (grass fields; 87%).

In the Netherlands, 57 queens were encountered nest searching during random transect walks. A hole in the ground or small patch was investigated by a queen during 38 of these observation periods. Most queens were seen in the 'Heathland' habitat type and no queens were seen exhibiting search behaviour in the grass area reserved for dog walkers ('Grass/dog play area'). Significantly more queens were observed nest site searching in the 'Mixed forest' land classification than would be expected if distribution was only based on land cover proportion (chi^2 test: $p = 0.024$, total $chi^2 = 13.36$, d.f. = 11.07; *Table 1*).

In New Zealand only 12 queens were encountered with ground level investigations occurring on four occasions. Most queens were seen in 'Open farmland', six of the 12, and no queens were observed in 'Deciduous forest', 'Mixed forest' or areas with anthropological features. Due to the small size of the New Zealand sample no further analyses were conducted.

Table 3.1: Results of chi^2 analysis carried out on the expected and observed numbers of queens seen in each habitat type in the Netherlands (n=59, two of the 57 queens spent equal time in two different classification types). Expected variables generated using the proportion of the total site each habitat type fills on the map created in ArcMap. P values significant >0.05.

Habitat Type	**Expected**	**Observed**	**Chi^2 Stat**	**P Value**	**Significant?**
Deciduous forest	4	9	2.1612	0.1415	No
Evergreen forest	10	6	1.1569	0.2821	No
Grass/Dog play area	3	0	3.0783	0.0793	No
Heathland	30	25	0.8514	0.3562	No
Mixed forest	5	14	5.0813	**0.02419**	Significant
Mowed heath	4	4	0	1	No
Path	3	1	1.0351	0.309	No
			Total chi^2 = 13.364	Degrees of freedom =11.07	

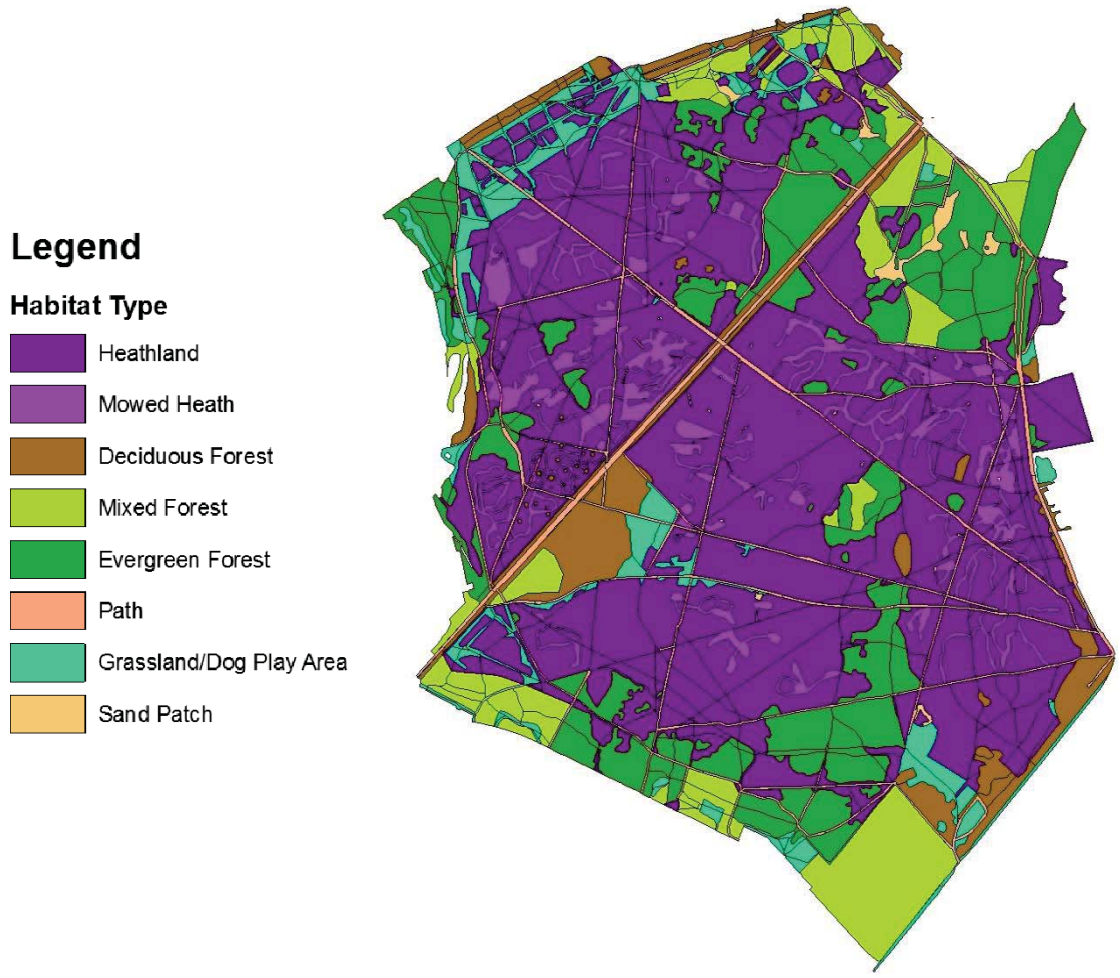

Figure 3.3: map of the Goois Natuurreservaat generated using the GIS software ArcMap to establish the area each habitat type covers for the field site.

Figure 3.4: map of the farm used as a field site in the Manawatu, New Zealand, generated using the GIS software ArcMap to establish the area each habitat type covers.

In most instances, the actual proportion of land each habitat type covers (determined in ArcMap) and the distribution of the random vegetation surveys were well aligned. This causes instances where the observed number of queens (represented in red bars) differs to stand out in Figures 3.5 & 3.6. One such instance is the discrepancy between 50% of the land area being defined as 'Heathland' in both ArcMap and the vegetation surveys in the Netherlands but only 42% of queens being observed here. The number of queens seen in deciduous and mixed forest also stands out and, as mentioned previously, the inconsistency was found to be statistically significant for 'Mixed forest'. In New Zealand also, despite the small sample size, some clear differences emerge. The two most obvious of these are the high percentage of queens seen in evergreen forest (33.3% observed, 6.5% expected) and the disproportionate number of queens seen searching in open fields (50% observed, 87% expected).

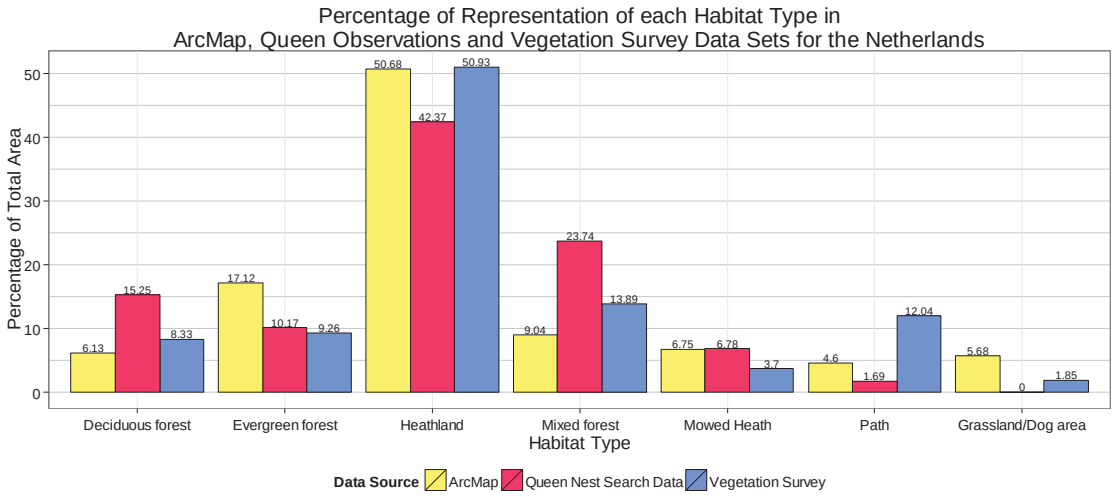

Figure 3.5: Comparing the percentage of each habitat type's distribution (based on ArcMap habitat map) to where queens were observed searching and where the random vegetation surveys were conducted in the Netherlands. The habitat distributions calculated in ArcMap served as expected variables for further analyses.

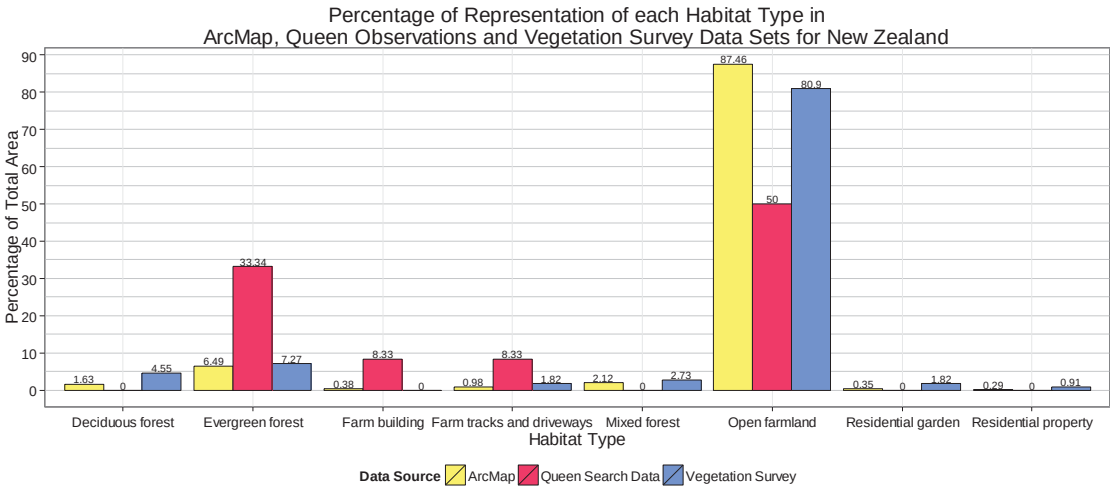

Figure 3.6: Comparing the percentage of each habitat type's distribution (based on ArcMap habitat map) to where queens were observed searching and where the random vegetation surveys were conducted in New Zealand. The habitat distributions calculated in ArcMap served as expected variables for further analyses.

In the Netherlands information was gathered about the direction any hole investigated by a queen was facing if it didn't open in a vertical fashion (*Figure 3.7*). Over 30% of holes were pointed in a southerly direction, followed by almost 23% facing west. The prevailing wind in the study area is southwest which coincides with the general direction most holes were facing.

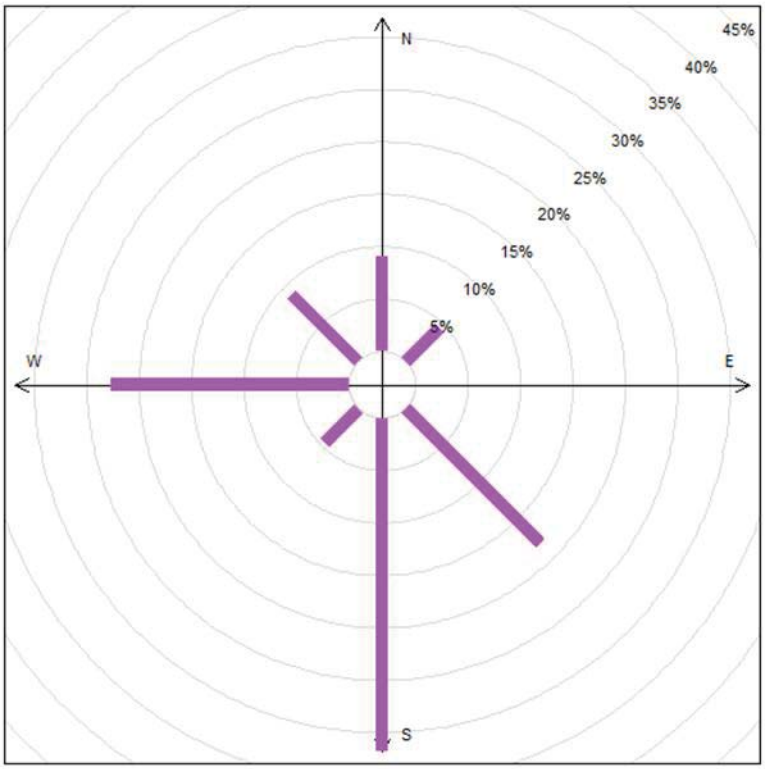

Figure 3.7: percentage of holes investigated by B. terrestris queens facing each of the eight key compass bearings in the Netherlands. Twenty-two holes were recorded.

The majority of the vegetation surveys (37.4%) and points of investigation (35.9%) occurred at places where moss was the main ground cover and therefore the most prominent component in the top soil layer (*Figure 3.8*). While 20.6% of vegetation plots had a moss and topsoil groundcover, just 2.6% percent of queens were observed searching areas with the same groundcover. The opposite relationship was seen for places that had a leaf litter and moss soil type combination: only 6.5% of plots were covered in leaf litter and moss, but 23.1% of investigation points were observed here (*Figure 3.8*).

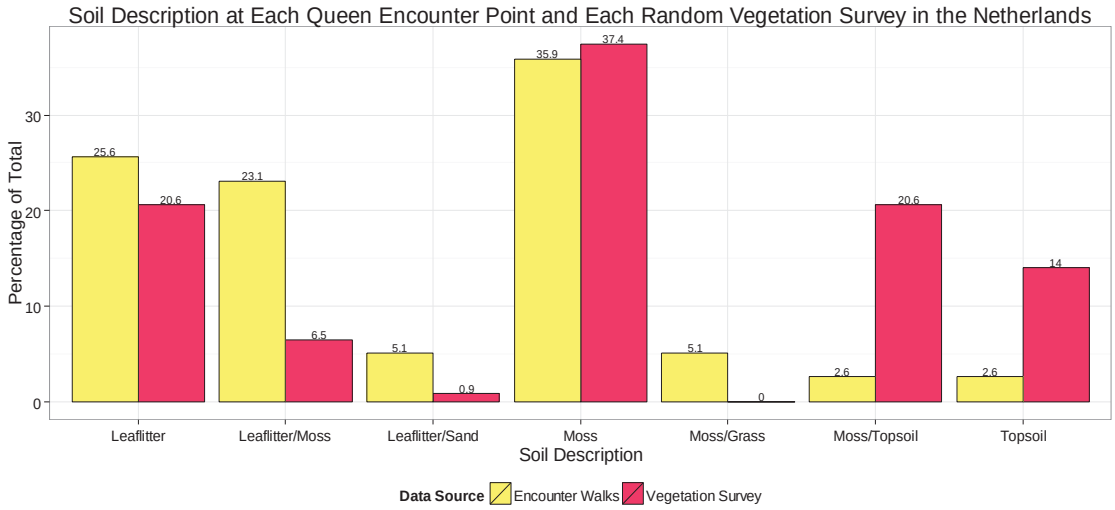

Figure 3.8: comparing the proportion of times each soil type was encountered by B. terrestris queens nest searching and during the randomly distributed vegetation surveys at the Netherlands' field site.

Discussion

The desired outcome of the research in both New Zealand and the Netherlands was to find evidence of any innate preference to search for nest sites in particular areas. Those queens seen did appear to search for nesting sites under the cover of trees preferentially. Analysis of the number of queens observed searching in mixed forest plots in the Netherlands was found to be statistically significant. Unfortunately the very small number of queens observed in New Zealand (n=12) did not provide enough data for statistical testing. There did however seem to be a disproportionate number of queens seen in evergreen forest, four out of twelve, compared to how much of the study site consisted of evergreen blocks: 6.5%.

A preference for nesting under trees could be explained in a number of ways: the trees would provide more shelter for a nest than for one established in open grassland (Kells & Goulson, 2003), they stand out as strong markers in the landscape for relocating the nest entrance after a forging bout (Osborne, 2008), or the leaf litter covering the ground under trees may be especially appealing to queens.

Some evidence in support of the latter of these explanations has been found in the present study. The number of sites in the randomised vegetation surveys in the Netherlands in which the top soil layer contained leaf litter was approximately 28 (that is, only 25%) but queens were observed searching in areas with that same soil description almost 50% of the time. To be more precise, a mix of leaf litter and moss was the most preferred with over 15% more queens seen searching in that classification than expected. Moss was very common in the nature reserve often forming blankets over the ground in the heathland and growing amongst the leaf litter under trees.

Queens were least likely to be seen nest searching in places with bare ground or short grass in the Netherlands and investigated holes facing a north to east direction rarely.

This could have something to do with the sun tracking the southern side of the sky in spring in the Northern Hemisphere. Holes facing south would be exposed to a greater amount of solar radiation making them warmer and dryer nest entrances.

Queens were observed less often in open expanses of grass in New Zealand than would have been expected if search behaviour was random. The small sample size in New Zealand makes any conclusions tentative but the method of comparing data from queens encountered through random transect walks to the exact habitat distributions is still favourable as it removes bias that may be present in studies using pre-determined transects (Kells & Goulson, 2003; Svensson *et al*, 2000). The random vegetation surveys also served their purpose well, lining up with the actual distributions on most occasions and providing more informative data like soil type that could not be gathered from aerial photography.

It has previously been observed that some bumblebees in New Zealand will establish nests throughout the year rather than entering a hibernation period over winter (Cumber, 1954). This may be one explanation for the low number of queen sightings here. The present study was conducted at the beginning of spring in both locations and in the Northern Hemisphere this is when an influx of queens will emerge from hibernation. This same influx may not have occurred in New Zealand.

It is also possible that there was a lower density of *B. terrestris* queens at the New Zealand site than the Netherlands, decreasing the chance of encountering a queen nest searching. More time spent conducting the transect walks or more researchers working at the site may have countered this but that was not feasible in the present study.

The aim of this study was to uncover *B. terrestris* nesting preferences that could be applied to the design and placement of domiciles in New Zealand and inclination to nest search under the cover of trees was apparent at both study sites. It would certainly be interesting to trial placement of domiciles under forest blocks in New Zealand with a leaf-litter ground covering to see if a higher *B. terrestris* acceptance rate can be achieved than in previous research. This could be done in orchards by placing domiciles under windbreaks or in nearby wooded areas. Growers could also see if supplementing the ground cover over and around buried domiciles with extra leaf litter further increases nest establishment rates. There is a large scope for further research though which may enable a more refined picture of where queens will nest in New Zealand.

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Chapter Four

Finding Early Stage *Bombus terrestris* Nests Using Radio Telemetry Tracking

Abstract

The nest entrances of ground dwelling bumblebee species such as *Bombus terrestris* are often well concealed and infrequent forager traffic makes them difficult to locate by eye. This makes studying the species' nesting behaviour difficult and the current knowledge base is not extensive. The present study used radio telemetry tracking of queen bumblebees to try and locate early stage *B. terrestris* nests. Two weeks were spent at field sites in both the Netherlands and New Zealand in the middle of the spring season. Five queens were successfully tagged with miniaturised radio transmitters (0.2 grams) in the Netherlands but no queens were able to be tagged in New Zealand. Of the five queens tagged only one led researchers to a nest site. Despite the lack of success in this study, radio telemetry tracking of queens is still a promising methodology for locating nests and the outlook is bright as technology advances in the industry.

Introduction

Locating the nests of *Bombus* species in the wild is a difficult task. Forager traffic to and from nests can be infrequent making nest entrances, which are often at ground level, hard to find (Kells & Goulson, 2003). As a consequence, relatively little is known about the specific nesting habits of economically important bumblebee species (Lye *et al.*, 2012).

Understanding the nesting preferences of abundant species could prove very useful for developing ways to increase nest density in orchards and fields and consequently improve the pollination service provided by bumblebees. Artificial nesting domiciles have and continue to be trialled in a variety of designs and locations as a way to facilitate an increase in bumblebee numbers. Large variations in success between species and locations have been reported (Fye & Medler, 1954; Hobbs *et al*., 1960; Lye *et al.,* 2011). In New Zealand there have been frustratingly low uptake rates for the most common species, *Bombus terrestris* (Barron *et al.,* 2000; Donovan & Wier, 1978).

If more information was available about what attracts *Bombus terrestris* queens to their natural nesting sites then this could be applied to the design and placement of domiciles and may lead to them being accepted as suitable nesting sites more frequently. Colonies established in domiciles can be closely monitored which may allow for supplementary feeding and control of pests and diseases if needed. An increase in the health of hives is likely to lead to more reproductive bees being produced at the end of each cycle (Suzuki *et al*., 2009) and a flow on effect of higher local nest density in subsequent seasons.

Modern technology can potentially overcome barriers that would otherwise limit research, such as the difficulty in locating nests by eye. The miniaturised radio transmitters used in radio telemetry have advanced to a stage where they are small enough to be used on large insects like scarab beetles, katydids, dragonflies and bumblebees (Kissling *et al.,* 2014).

Hagen *et al.* (2011) conducted the first study using radio telemetry on bumblebees, investigating the spatial-temporal movements of three species, including *B. terrestris*. The present study aims to determine the location of early *B. terrestris* queens' nests using the same radio telemetry tracking method as Hagen *et al*. to increase knowledge of what queens qualify as a suitable nesting site. If this aim is achieved successfully then the information has the potential to be applied to domicile design and placement in New Zealand.

Methods

Study Time and Location

The first study took place over two weeks from April 14th – April 28th 2014 in the Goois Nature Reserve in the Netherlands (52.254098 latitude, 5.189581 longitude). The site is a 4.7km^2 expanse comprising of open heathland with patches of broom, *Cytisus scoparius* (a key forage plant for emerging queens in this area), and forest blocks.

The second study was conducted during October 2014 on a farm in the Manawatu region of New Zealand (-40.288876 latitude, 175.647912 longitude). This site is 3.6km^2 and is mostly open grass fields but also contains deciduous and evergreen forest blocks as well as some anthropological features like large hay sheds, concrete pads and houses.

Study Subjects

We targeted *B. terrestris* queens that were collecting pollen in their corbiculae, as this indicated that a nest site had been established and provisioning had begun. Queens were caught from flowers during foraging bouts and tracked back to their nests using miniaturised radio transmitters. The body length of each queen was measured while she was being held still for transmitter attachment.

Radio Transmitter Attachment

The transmitters used were the A2412 model from Advanced Telemetry Systems in Minnesota, USA. They each weighed 0.2 grams and measured 12 x 5 x 1.5 mm. Prior to being taken in the field, transmitters were prepped by checking the battery and confirming that a strong signal was being emitted. The best receiving frequency was also determined. The transmitters had their antennae cut to 3cms in length and were wrapped in a thin layer of Parafilm which allowed easier removal from the queens if needed and prevented glue from coming into contact with the battery.

Transmitters were attached to the abdomens of the queens selected for tracking. In order to allow transmitter attachment, a *B. terrestris* queen would be placed inside an empty 50mL plastic syringe that had the top cut off and a layer of thin mesh secured in its place by rubber band. The rubber plunger of the syringe also had a layer of cotton wool glued on top to provide a cushioned surface for the bumblebee during transmitter attachment.

Once the queen was inside the tube and sitting flat on the cotton wool, the plunger would be pushed up until she was held firmly in place beneath the mesh covering. A scalpel was then used to cut open a small section of the mesh over the queen's abdomen. Pins were used to hold her wings back off her abdomen and clear of the glue. The scalpel was then used to shave off a small patch of hair from the top of the abdomen about 7mm long and 4mm wide. A small drop of Bostik brand Super Glue was then placed on the bare exoskeleton and another drop on the flat base of the battery component of the transmitter. This was left to air-dry for about 15 seconds then the

transmitter was placed on the abdomen and held in place for 1 minute to allow the glue to set completely.

Once the transmitter felt as though it was firmly in place the queen was released into a small container and monitored for any signs of wing damage or low energy. If she did appear very slow then an attempt was made to feed sucrose water. When researchers felt confident the transmitter was secured and the queen appeared fit to fly she was released back onto the same forage patch she was caught on.

Telemetry Tracking

Immediately after attaching the transmitter, tracking could begin and previously tagged queens could be tracked at any time. A three element yagi antenna or a small dipole antenna would be attached to the receiver unit and the frequency of interest dialled in. Tracking would begin at the last known location of the individual.

When a signal was received the location of the queen would be sought by following the bearing that the strongest signal was coming from. The signal from the yagi antenna would increase or decrease in strength depending on how close to the queen's location the antenna was pointed.

When two researchers with directional yagi antennae were present, queens could be tracked via triangulation. This involved slowly closing in on the point where the strongest signals they each received crossed. Once a queen had been found, if she wasn't already at her nest site, researchers would try and follow her until she returned to her nest.

Data Collection at Nest Sites

When a nest entrance was located the following information was collected about the location and surroundings:

- Nest search GPS number
- Start Time
- Area Searched
- Plot Description
- Direction of Hole
- Aspect
- Notes
- Date
- End Time
- General Description
- Soil Description
- Slope
- Nearest Tree

Results and Discussion

The Netherlands

At the Netherlands study site, five queens were successfully tagged (*Figure 4.1)*. Unfortunately, Queens four and five were tagged in an area of the site which was quite close to a large radio and television tower that constantly produced 'noise' in the receivers used for telemetry. This made tracking the queens extremely difficult and locating them after the first day was not possible. No nest site information was collected from these queens.

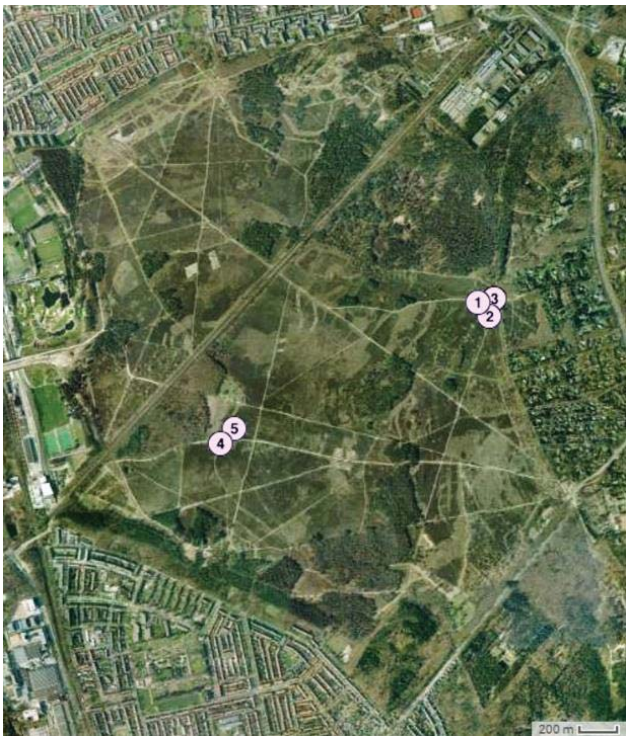

Figure 4.1: Map of the Goois Nature Reserve in the Netherlands showing the locations where five queens tracked via radio telemetry were caught.

Queens one, two and three were all caught on a large patch of flowering Broom (*Cytisus scoparius)* on the eastern edge of the site. Queen three was not located again after the first day of tracking and no nest was recorded. Queens one and two were able to be tracked on successive days but it is suspected queen two eventually left the field site and no nest information was collected. Queen one however did lead the researchers to a hole in a bank which she returned to several times so it was concluded this was her nest site.

The site was under large oak trees and the ground cover consisted of moss, leaf litter and short grass. The ground was sloping in an easterly direction but the entrance hole opened upwards.

New Zealand

No queens were successfully tagged or tracked at the New Zealand study site during the October study period. *B. terrestris* queens carrying pollen were seen only 4 times at the forage sources which were frequented by workers and other bumblebees and despite repeated attempts none were able to be fitted with a radio transmitting device.

A possible explanation for this is that there is a low density of *B. terrestris* queens establishing nests at the start of spring due to the mild winters in the Manawatu. The average winter temperature for this region is approximately 13ºC, compared to the study site in the Netherlands which has an average winter temperature of approximately 6ºC. Fertile queens either do not enter hibernation in autumn or emerge from hibernation earlier than in the European season. This mean nests are able to be established at any time of the year, as observed by Cumber in 1954, so there is not the same springtime influx of nest searching queens as is observed in the Northern Hemisphere.

Transmitter Effect on Queens

In this study, the five queens which had a transmitter successfully attached showed differences in their behaviour soon after release. Two remained at their release locations cleaning themselves and resting before flying away; the same behaviour was observed by Hagen *et al* 2011. Another flew about 40m from her release location straightaway and landed high in a tree out of sight where she stayed for approximately 30 minutes. Another queen was tracked flying distances between 10-50m at a time, landing briefly between each flight to groom herself or rest before flying in to a residential garden where she could no longer be tracked. One queen almost immediately flew a large distance (>300m) quickly towards to edge of the field site which led the researcher's to lose track of her. It is believed she entered the residential area where tracking was not possible.

In Hagen *et al's* 2011 paper an investigation was conducted on the effect of transmitter attachment to bumblebees and there was some indication that transmitters do incur significant energy costs. They recorded long resting periods (over 45 minutes) between flights for a *B. hortorum* individual as well as lower flower visitation rates and a longer length of time spent on each flower head for workers of *B. terrestris* with a transmitter compared to those without.

The individuals used in this study were all queens, however, which do display dimorphism from workers in that they are significantly larger. For example, queens of species *B. haemorrhoidalis* have an average body length almost a centimetre longer than that of workers; 2.6cm and 1.8cm respectively. The queens tagged in this study ranged in length from 2.27cm to 2.76cm (average length 2.5cm). We can infer from past research that queens are able to carry heavier loads of pollen and nectar than workers and the same should apply to the radio transmitters. More research is needed in this area though to confirm the energetic costs of transmitter attachment on queens.

Limitations and Future Directions

Several limiting factors hindered the success of this study. If more time or a larger team of researchers was available to carry out the telemetry then there may have been more nests found. Environmental constraints were also an issue such as bad weather reducing queen abundance and cold days causing their body temperature to drop rapidly during handling, possibly impacting on their flight ability when released. The size of the transmitters relative to the bees made attachment challenging. If the size of the smallest transmitters can be reduced even further then the time from catching to tracking could be reduced which would be advantageous.

The use of telemetry in studies of bumblebees does have potential but unfortunately it did not prove to be very effective in this instance. Only one nest site was located in over four weeks of investigation across two field sites. It is possible more nests could have been found in the same amount of time using a different methodology.

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Chapter Five

General Conclusions and Future Directions

Revising the Distribution of *Bombus hortorum* in New Zealand

Revision of the distribution of *Bombus hortorum,* which shares a cryptic morphology with *B. ruderatus* in New Zealand, using primarily citizen science to gather samples was successful. New location data for the species in Napier, Taihape and the Waikato region was recorded. Digestion of PCR products using the Tsp45I restriction enzyme allowed species determination via the presence of one (undigested) or two (digested) bands of DNA representing *B. ruderatus* and *B. hortorum* respectively (Ellis *et al*, 2006). The new information tentatively places an updated maximum distribution line over 250km farther north than previously reported in the literature (Macfarlane & Gurr, 1995; Howlett & Donovan, 2010).

The result is tentative as no samples were sent in from the Auckland region so the range of *B. hortorum* could extend farther than was able to be ascertained. For future studies a more targeted approach could be taken when seeking specimens. Local newspapers, radio stations or websites in areas of interest could be used to advertise the study rather than the more generalised approach that was taken here. This would allow a more detailed distribution map to be built.

Following the success of the HotSHOT DNA extraction protocol (Truett *et al*, 2000) and Tsp45I restriction enzyme for identification of fresh specimens, an attempt was made to replicate the methodology on museum specimens. Results were suboptimal in that instance. Only eight of 26 museum specimens ranging in age from 1953-2013 had DNA extracted and then amplified by PCR. The digestion procedure was carried out on those eight amplifications but only four yielded visible bands on a 3% agarose gel (1953, 2009, 2010 and 2013).

Of these four visible banding patterns, two contained three bands each: 2009 and 2010. This has been attributed to incomplete digestion previously (Ellis *et al*, 2006) but in this study it was revealed by sequencing to be caused by the presence of two distinct haplotypes. One version contained the restriction enzyme cutting site and one did not, meaning the three bands persisted even after exposure to more enzyme.

The source of the mixed haplotypes is unknown but it prevented a species being assigned to the two specimens of interest, calling into question the efficacy of this identification tool. One possible explanation is that hybridisation has occurred between the two cryptic bumblebee species in New Zealand, *B. hortorum* and *B. ruderatus*. This break down in species integrity is not unheard of as hybridisation events have been identified between *B. ruderatus* and another bumblebee in the same morphological grouping in France (Scholl *et al*, 1992). Further research should be pursued to determine whether or not hybridisation is occuring in New Zealand.

The disappointingly low number of successful PCRs from museum specimens prompted comparing the HotSHOT protocol's extraction ability to a DNeasy Kit (Qiagen©) and a 'Salting Out' technique (Sunnuck & Hales, 1996) using 16 bees from Massey University's collection. Each bee had three legs removed allowing all three extractions

to be trialled on all specimens. This process provided more evidence to suggest the HotSHOT protocol is not well suited to extracting DNA from museum specimens.

All extractions were put through the NanoDrop 1000 Spectrophotometer and although the HotSHOT protocol had the highest average extraction concentration (427.8 Ng/µL) it had the lowest average 260/280nm and 260/230nm absorbance ratios (1.548 and 0.739), indicative of some kind of contamination (Glasel, 1995). This contamination may be inhibitive during the PCR process preventing a strong amplification.

It is somewhat unsurprising that the HotSHOT protocol will result in high levels of contamination as everything added during the extraction remains in the final product. This includes fragments of bumblebee cuticle and hairs. The presence of these may be falsely contributing to the high concentration reading. It is recommended that future studies quantify DNA concentration using a Qubit Fluorometer which has the ability to determine the quantity of DNA specifically and won't provide confounded readings in the presence of other bio-molecules.

More time should also be spent testing the strength of each extraction method when a longer period is spent in the cell breakdown stage and a larger initial tissue sample is added. This could greatly increase the chance of obtaining PCR quality DNA from dried museum specimens. The tendency of the HotSHOT protocol to shear DNA fragments in to shorter lengths (Truett *et al*, 2000) may hinder it ever becoming a suitable technique for museum specimens though, which likely contain DNA that has already degraded in to a fragmented state.

Investigating the Nesting Behaviour of Bombus terrestris in New Zealand and the Netherlands

The method of encountering nest searching queens by chance during random transect walks allowed observations of 57 *B. terrestris* queens to be collected in the Netherlands. From these observations it became evident that the queens showed a preference for searching under deciduous and mixed forest blocks and appeared to avoid open areas such as paths or very short grass. The number of queens seen searching under mixed forest was deemed statistically significant ($p = < 0.05$). In addition to a land use type preference, they also searched more frequently in places with a moss and leaf litter ground cover.

In New Zealand the methodology led to 12 queens being encountered in the same amount of time, a substantially smaller number. The lack of samples meant statistical significance testing could not be carried out but the data did suggest that a preference for searching under evergreen forest was emerging. Similar to the Netherlands, the queens appeared to show an avoidance of large open areas preferring to be undercover.

This level of depth in investigating the specific nest search habits of *B. terrestris* queens could lead to an increase in acceptance of domiciles when the relevent characteristics are applied to their design and placement. A larger sample of queens from New Zealand was certainly desirable though as the demand for increased nest densities in orchards

and crops is high here but domicile acceptance by *B. terrestris* is low (Donovan & Wier, 1978; Barron *et al*, 2000). More observations would have allowed a greater resolution to be achieved when considering differences between nesting habits in New Zealand and Dutch populations.

The lower number of queens seen may be due to a smaller population density at the New Zealand site, subsequently reducing the chance of encountering a queen. Further research in to the nesting behaviour of bumblebees in New Zealand is recommended and it would be advantageous to have multiple researchers walking different transects at the same time in future studies. This should increase the number of queens observed over all.

Despite the limitations, the use of random transects to encounter queens is still advised because of their lack of bias. The use of the GIS software ArcMap, as well as random vegetation surveys across the landscape, provided suitable information to compare the queen observations to so it is also suggested their use continues in future work.

Finding Early Stage Bombus terrestris Nests Using Radio Telemetry Tracking

Locating early nests of *B. terrestris* queens using radio telemetry tracking is a promising option but was not very effective in this study. Five queens were tagged with miniturised radio transmitters at the Netherlands field site but only one led researchers to a nest discovery. No queens were tagged at the New Zealand study site.

The process of finding a queen, tagging her and then tracking using directional antennae was time consuming and to have found only one nest in a total of four weeks dedicated telemetry work makes the methodology very inefficient.

It has been suggested that the attachment of the 0.2g transmitter (Advanced Telemetry Systems) may negatively affect bumblebee foraging and flight effeciency (Hagen *et al*, 2011). Large variations in the behaviour of queens were observed immediately after release in this study, ranging from queens remaining at their release site for up to 30 minutes to a queen who flew over 300m straighaway, but the small sample size means it was not possible to quantify these differences.

As technology continues to advance, the constraints around transmitter size and weight will likely be reduced allowing for easier attachment to queens (and other insects) and a lessened impact on foraging and flight behaviour. The development of automated tracking systems would also make this method more feasible as multiple queens could be tracked simultaneously and researchers could dedicate their time to tagging rather than manual tracking. More indepth information about the movement of queens could be recorded by an automated system revealing a lot more about *B. terrestris* nest selection behaviour than is currently known.

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Appendix 1 - Letter Requesting Bumblebees

Katie Ashley
Plant & Food Research
Bisley Road
Ruakura
Hamilton

To Whom It May Concern,

My name is Katie Ashley and I am studying bumblebees for my MSc thesis through Massey University. Alastair Robertson is my principal supervisor, and my co-supervisor is David Pattemore from the Pollination and Apiculture team at Plant & Food Research in Hamilton. My project is part of a wider six-year research programme on bumblebees in NZ.

For one aspect of my thesis I am interested in generating an updated map of species distributions throughout New Zealand. It has been almost 20 years since this was last done and using modern genetic techniques means far greater accuracy can now be achieved.

There are four bumblebee species currently known to be present here as a result of introductions from England around 1900. Two of these, *B. ruderatus* and *B. hortorum*, have very similar morphology and markings (two yellow stripes on the thorax) and this means they can easily be confused. In the UK, genetic analysis is now being used to distinguish the two species and we wish to use that same method here.

I am seeking specimens of these 'two striped' bumblebees from all regions of New Zealand to carry out this genetic analysis. If you see any bumblebees that look like those in the images supplied please catch them, place them in the freezer overnight (this is a common and well accepted method of euthanizing insects) and send them to me at the following postal address (courier address at top of page, also c/o David Pattemore):

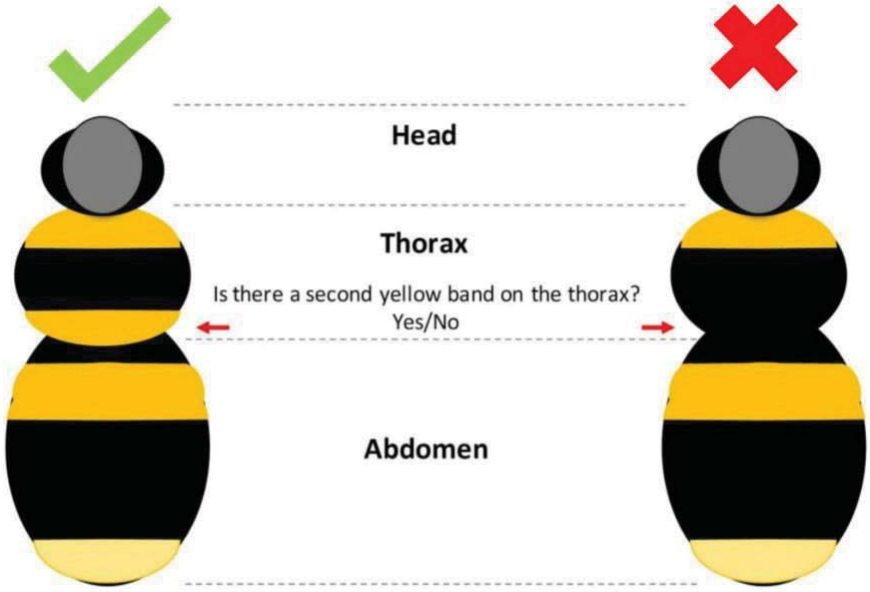
Katie Ashley c/o David Pattemore
Private Bag 3230
Waikato Mail Centre
Hamilton 3240

Extra information:

- When collecting specimens, it is possible that all bees within relatively close proximity are from the same hive so it is a good idea to only collect a few from each area they are seen in.
- As the hive grows, workers produced become bigger so a small bee and a rather large bee in the same area could be from the same hive. Even the smallest bees can be used for genetic studies so don't worry about size when collecting.
- Take care when collecting specimens, they can sting!
- It is possible for these bees to be coloured very dark or completely black; these specimens are still useful for genetic testing. If in doubt, send it in!
- Please include the most accurate address possible for where the bee was caught as well as a brief description of what it was doing at the time i.e. foraging on agapanthus, crawling across pine needles in leaf litter, flying over lawn, etc.
- Absolutely any location in New Zealand is suitable for this collection.

If you have any questions about the content of this letter or the collection process, please do not hesitate to contact me at katie.ashley99@gmail.com.

Many thanks



If the bee clearly has only one yellow band on the thorax then it is *Bombus terrestris* and is not required for this collection.