#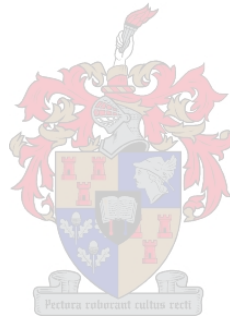 Status of the invasive wasp species, *Vespula germanica* and *Polistes dominula* in South Africa, and the feasibility of various management strategies

by

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Declaration

By submitting this thesis/dissertation electronically, I declare that the entirety of the work contained therein is my own, original work, that I am the sole author thereof (save to the extent explicitly otherwise stated), that reproduction and publication thereof by Stellenbosch University will not infringe any third party rights and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

December 2016

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Abstract

The main objective of this study was to determine the current status of two invasive wasps, *Vespula germanica* and *Polistes dominula,* in South Africa and to explore the feasibility of implementing various management strategies to control and/or eradicate them. Both wasp species pose a potential threat to biodiversity and agriculture in the Cape Floristic Region (CFR), as well as being a nuisance to people.

In an effort to identify suitable biocontrol agents, the pathogenicity of three likely indigenous entomopathogenic nematode (EPN) species and one likely indigenous entomopathogenic fungal (EPF) species was tested against both *V. germanica* and *P. dominula* larvae in a bioassay trial. The three EPN species were *Heterorhabditis bacteriophora*, *H. noenieputensis* and *Steinernema yirgalemense.* The fungal species tested was *Beauveria bassiana.* Both *V. germanica* and *P. dominula* larvae were highly susceptible to all of the biocontrol agents tested, and died of infection within 4 days after inoculation with EPN and within 7 days after inoculation with the EPF.

The EPN *Heterorhabditis bacteriophora* which performed best in the bioassay trial, as well as the EPF, *Beauveria bassiana,* were then tested in the field to determine its ability to infect *P. dominula* larvae by spraying inoculum directly onto nests. Four treatments were applied, namely: an aqueous solution of the EPF, an aqueous solution of the EPN, a mixture of the EPF and EPN species, and a control of distilled water. The combination of EPF and EPN caused the highest mortality in both *P. dominula* larvae (31.39 %) and pupae (3.42 %) compared to the other treatments, but infection levels were much lower than those obtained under laboratory conditions. An unsuspected discovery was made, when it appeared that 13 % of all nests used in this trial were parasitized by an unclassified fly species, identified to be a species from the Tachinidae family. There was no significant difference between the ability of fly larvae that were treated with the control, to develop into adults over a period of 144 h, compared to those treated with the various biocontrol agents.

Landmark-based geometric morphometric analyses were used to identify the potential origin of introduced *V. germanica* wasps and to determine the possible route of invasion followed in South Africa. Variation in forewing shape among wasp worker samples that were collected from five different countries, including South Africa, Australia, New Zealand, Argentina and France, was compared. An overall direct correlation between the wing shape and the geographic distance between two sites was found. This result suggests that the morphological variation in wasps from South Africa can be explained as isolation-by-distance. Results inferred that wasps had spread from Kirstenbosch to Somerset West, and thence through Stellenbosch to Franschhoek. The wing shape of wasps collected

from Kirstenbosch, the area where the first *V. germanica* specimen was found, mostly resembled the wing shape of samples from France, compared to all the other overseas localities. Therefore, one could conclude *V. germanica* wasps were most likely transported from Europe to South Africa.

The attractiveness of a range of lures and baits to *V. germanica* and *P. dominula* females collected in the field were tested using a Y-tube olfactometer. A combination of protein and carbohydrate-based baits were tested. *Vespula germanica* mostly preferred cooked ham, whereas *P. dominula* was mostly attracted to the odours emanating from their own nest.

Uittreksel

Die hoofoogmerk van hierdie studie was om die huidige status van twee indringerwespe, *Vespula germanica* en *Polistes dominula,* in Suid-Afrika te bepaal, en om die raadsaamheid te ondersoek om bestuurstrategieë toe te pas om die wespe te beheer. Albei spesies wespe hou 'n potensiële bedreiging in vir biodiversiteit en landbou in die Kaapse Floristiese Streek (KFS), en hulle is 'n ergernis vir mense.

Ten einde geskikte agente vir biokontrole te identifiseer, is die patogenesiteit van drie spesies waarskynlik inheemse entomopatogeniese nematode (EPN) en een spesie waarskynlik inheemse entomopatogeniese fungus (EPF) getoets in 'n bio-essaiproef op die larwes van sowel *V. germanica* as *P. dominula*. Die EPN spesies was *Heterorhabditis bacteriophora*, *H. noenieputensis* en *Steinernema yirgalemense,* en die fungusspesie *Beauveria bassiana.* Die larwes van sowel *V. germanica* as *P. dominula* was hoogs vatbaar vir al die biokontrole-agente in die proef, en het binne 4 dae na inenting met die EPN, en binne 7 dae na inenting met die EPF, van infeksie gesterf.

Die EPN *Heterorhabditis bacteriophora*, wat die beste in die bio-essaiproef gevaar het, sowel as die EPF, *Beauveria bassiana,* is daarop in die veld getoets deur entstof regstreeks op die neste te spuit om hul vermoë te bepaal om die larwes van *P. dominula* te infekteer. Vier behandelings is toegedien, naamlik: 'n water-oplossing van die EPF, 'n water-oplossing van die EPN, 'n mengsel van die EPF- en EPN-spesies, en 'n kontrole van gedistilleerde water. Die kombinasie van EPF en EPN het die hoogste sterfte veroorsaak in sowel *P. dominula* se larwes (31.39 %) as papies (3.42 %) in vergelyking met die ander behandelings, maar die vlakke van infeksie was heelwat laer as wat onder laboratoriumtoestande verkry is. 'n Onverwagte ontdekking is gedoen, toe dit blyk dat 13 % van alle neste wat in hierdie proef gebruik is deur 'n ongeklassifiseerde vlieg-spesie, van die familie Tachinidae, geparasitiseer is. Daar was geen noemenswaardige verskil tussen die vermoë van vlieglarwes wat behandel is met die kontrole en dié behandel met die onderskeie biokontrole-agente, om in volwassenes te ontwikkel oor 'n tydperk van 144 h nie.

Ten einde die moontlike oorsprong te bepaal van ingevoerde *V. germanica* wespe, asook die moontlike roete van indringing wat in Suid-Afrika gevolg is, is oriëntasiepunt-gegronde geometriese morfometriese analises gedoen. Verskille in die vorm van voorvlerke is vergelyk tussen voorbeelde van werkerwespe wat in vyf verskillende lande versamel is, waaronder Suid-Afrika, Australië, Nieu-Seeland, Argentinië en Frankryk. 'n Algehele regstreekse korrelasie is gevind tussen die vlerkvorm en

die geografiese afstand tussen twee plekke. Hierdie resultaat dui daarop dat morfologiese afwykings tussen wespe in Suid-Afrika verklaar kan word as isolasie-deur-afstand. Uit die resultate kan afgelei word dat die wespe van Kirstenbosch na Somerset-Wes versprei het, en vandaar deur Stellenbosch na Franschhoek. Die vlerkvorm van wespe wat in Kirstenbosch versamel is, die gebied waar die eerste *V. germanica* voorbeeld gevind is, het in vergelyking met die ander oorsese plekke, die meeste soos die vlerkvorme van voorbeelde uit Frankryk gelyk. Die gevolgtrekking kan dus gemaak word dat *V. germanica* wespe heelwaarskynlik van Europa na Suid-Afrika vervoer is.

Die aantreklikheid van 'n reeks lokmiddels en aas vir *V. germanica* en *P. dominula* wyfies wat in die veld versamel is, is in 'n Y-buisolfaktometer getoets. 'n Kombinasie van aas met 'n proteïen- of koolhidraatbasis is getoets. *V. germanica* het gekookte ham die meeste verkies, terwyl *P. dominula* meestal gelok is deur die geure vanuit hul eie nes.

Dedication

I dedicate this dissertation to

my family I could not choose, but would if I had to, over and over again.

my friends - I owe you my sanity.

the artists of glorious music, mediocre wine and transportive novels.

"It's turtles all the way down." - Uncertain

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Chapter 1

Literature review

Introduction

Introduced species are organisms that are neither indigenous, nor native to a specific location, and have been accidentally or deliberately moved to a new environment through human-mediated activity, breaching biogeographical boundaries to settle where they do not naturally occur (Richardson and Pyšek 2008; Sharp et al. 2011). Global trade expansion has led to an increase in the homogenization of species around the world. The redistribution of species currently occurs at a faster pace compared to previous decades and is so widespread that it constitutes a significant component of global environmental change (Soulé 1990; Vitousek et al. 1997; Meyerson and Mooney 2007). Not all introduced species become alien invasive species (AIS). Species that spread widely, and are self-sustaining in their new environment, are referred to as AIS. They become abundant and may out-compete, prey on, and hybridize with native species (Mooney and Cleland 2001; Jensen et al. 2005; Davis 2009). It follows that AIS could adversely impact the invaded ecosystem, causing environmental, social and economic harm (Pyšek and Richardson 2010).

The field of invasion ecology has piqued human interest for several decades. Since the publication of Charles Elton's (1958) foundational book, *The Ecology of Invasions by Animals and Plants*, academic interest and research contributions have surged (Richardson and Pyšek 2008; Jeschke et al. 2012). Invasion ecology is a modern, rapidly changing, interdisciplinary field of study with many of the principles in the field subject to ongoing debate. To better understand a field that is composed of complex biotic and abiotic interactions, with the goal of finding practical solutions to problems associated with the ever-increasing spread of species around the world, general principles and theories inform research and decision-making. That said, each invasion is unique, and should be treated as an individual case (Moller 1996; Pyšek and Hulme 2009).

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Biological invasions

There are various approaches and hypotheses in the discipline of invasion biology that explain the mechanisms involved (Jeschke et al. 2012; Masciocchi and Corley 2012). Three of the aspects that determine an invader's success are discussed in this study, namely: the characteristics of the invader, the characteristics of the invaded environment, and the interplay between the aforementioned two that may precipitate a specific outcome. The focus on each of these aspects will specifically concern dynamics associated with introduced social insects, which differ phenotypically from non-social insects to the extent that a different approach is required in analysing their invasions (Moller 1996; Heger and Trepl 2003).

Social invader species characteristics

One aspect that may predispose an insect species to become a successful invader is its specific life-history traits. The hypothesis of life-history traits having an influence on invasibility lends itself to be used as a predictive tool (Sakai et al. 2001). In general, the characteristics listed below illustrate a phenotypic plasticity that many non-invasive species do not possess (D'Adamo and Lozada 2009; Masciocchi and Corley 2012). High phenotypic plasticity enables social insects to adapt easily to new regions, because they essentially regulate the new environment in their favour to maximize their survival capabilities. This makes them notoriously successful invaders worldwide (Beggs et al. 2011; Rust and Su 2012).

Colony effect

Social insects, such as wasps, bees and ants, form large colonies, where specialisation of individuals and division of labour takes place. This is referred to as polyethism (Traniello and Rosengaus 1997). Many species of colony-forming insects are known to be extraordinary successful invaders representing a super organism (Gillooly et al. 2010). The colony allows them the flexibility to oscillate between individual and colony responses. This behaviour gives them an advantage to better withstand and buffer biotic and environmental resistances within an invaded community and can compensate for lack of individual plasticity (Beggs et al. 2011). The specialisation of workers who forage for resources, sidesteps the need for the foundress to leave the nest, which will expose her to predators (Spradbery 1973). Different foraging strategies among ant workers improve colony efficiency, where workers can, for instance, team up to kill prey (Sundström 1993). Specialised reproduction strategies,

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such as 'budding' in colonies of some honeybee and ant species, promote the establishment of new colonies. Cluster dispersal takes place as new foundresses are produced (Peeters 2001) while colony expansion evolves in the presence of conspecifics, rather than a solitary queen being solely responsible for the expansion. Individuals can also adjust work rate and life expectancy to the needs and size of a colony, for instance where overwintering wasp nests are controlled by more than one queen (Gambino 1991; Moller 1996).

Cognitive flexibility

The ability of social insects to learn, remember and integrate old and new experiences through visual and olfactory cues within new environments, contributes to their efficiency in locating food sources (D'Adamo et al. 2000; D'Adamo and Lozada 2007, 2011). Foragers are also able to recruit more foragers via behavioural or chemical communication.

Reproduction

Dispersing new queens mated at the end of the season can store sperm in spermatheca for prolonged periods whilst hibernating, which renders male wasp presence unnecessary once the queen emerges from hibernation. Stored sperm of wasps is genetically diverse, due to queens mating with several males (Loope et al. 2014). The reproductive rate of many wasps and ant colonies are high, as a result of short brood developmental time or high progeny production rate, which enables the production of large numbers of new queens to disperse and establish new colonies (Spradbery 1973; Farji-Brener and Corley 1998). Consequently, the impact of founder events such as population bottlenecks is minimized and several new populations can establish in a new environment (Fordham et al. 1991; Beggs et al. 2011).

Generalist predators

Many invasive insects incorporate a wide range of foods in their diet. Certain wasp and hornet species, such as *Vespula germanica* (Fabricius) (Hymenoptera: Vespidae), *V. velutina* (Lepeletier) and *V. vulgaris* (Linnaeus) have adopted a scavenger habit, which attracts them to non-living prey as well. This gives them an advantage over native wasps exhibiting a strictly predatory habit (Harris 1991; Richter 2000; D'Adamo and Lozada 2007; Villemant et al. 2011; Monceau et al. 2014). These evolutionary changes and habits, as well as habits such as foraging early in the morning, inadvertently

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extend the foraging time and season and make these invasive insects superior competitors (Edwards 1980).

Highly mobile

Social insects are highly mobile and have evolved to encourage dispersal, either passively or actively. They are strong fliers and can actively disperse up to 30-80 km (Masciocchi and Corley 2012). In order to invade new habitats, and to avoid catastrophic events and seasonal shifts, they mainly rely on passive dispersal aided by humans (Moller 1996). Passive dispersal can take the form of inseminated, hibernating queen wasps unintentionally transported among human goods to new environments, or dispersal of propagules and individuals via the wind (Porter and Savignano 1990; Liebhold and Tobin 2008).

Queen usurpation

Wasp and bee queens have the ability to parasitize established colonies, eventually displacing the colony's founding queen (Archer 1985; Spivak 1992; Schneider et al. 2004). The incoming queen colonises the nest by releasing a pheromone which blocks the sexual development of workers, who then resort to tending and raising of the new queen's offspring (Moller 1996). Cervo et al. (2000) hypothesised that reusing of old nests by *Polistes dominula* (Christ) (Hymenoptera: Vespidae) contributed to its successful establishment in new environments in the United States.

Polygyny

In some species of social insects, multi-queen nests occur, where nest mate queens exist as equals (Moller 1996). This is common in *P. dominula* as well as perennial *V. germanica* nests (Gambino 1991; Liebert et al. 2008). The number of queens also affects the production of sexuals, which in turn increases the number of new queen wasps to disperse and initiate colonies (Plunkett et al. 1989).

Body size

The body size of highly invasive species is said to contribute in part to their invasion success according to Ehrlich (1989), and, in many cases, large body size is a trade-off for high reproduction rates (Crawley 1989). However, social insects can produce large numbers of small solitary individuals to gather food, and the colony can defend itself as a large functioning unit (Ehrlich 1989; Moller 1996).

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Thus, the wasp colony acts as a super organism while still being small enough to consist of hundreds of individuals.

Longevity

In invaded environments where conditions can be suboptimal and temporally variable, long-lived invaders such as social wasps, fire ants and honeybee colonies prevail, because a successive cohort of individuals is produced over time, which eventually coincides with conditions conducive for the establishment of new colonies (Passera 1994; Moller 1996).

Naturalisation

Lee (2002) suggested that many species' invasion success might depend more heavily on genetic adaptation to the invaded environment than on physiological plasticity, which also applies to social insects. Many of the abovementioned traits characterise social wasps, which make them, unsurprisingly, the most successful invasive alien wasps (Beggs et al. 2011). Furthermore, specific intercorrelated characteristics might increase the potential of a species to be invasive, while the combination of other characteristics might reduce it (Sakai et al. 2001). Species characteristics are good indicators of invasiveness, but the traits of an invaded environment also play a role in explaining biological invasion processes.

Characteristics of an invaded environment

Studies by Crawley (1987) and Hengeveld (1989) suggest that all environments have the potential to be invaded, and the invasiveness of a species largely depends on its ecological plasticity and demography. The danger with that interpretation lies with the fact that conclusions are drawn from the highly reported outcomes of specific invasive groups, such as social insects, due to the conspicuous environmental harm they cause, their diurnal nature, and the fear that physical harm (stings) illicit in humans. Impacts associated with solitary or nocturnal species may then remain underreported or unnoticed (Moller 1996; Beggs et al. 2011) and, as a result, the mechanisms contributing to their invasiveness, as well. Moreover, some localities are not as thoroughly studied as others (Beggs et al. 2011). One species can establish, spread and become invasive in one location and not necessarily in another, even though similar traits are shared by the same species (Lockwood et al. 2013). Therefore, area-specific traits might be at play in determining invasion success.

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The first possible reason why some areas are severely invaded and others not, is that they engage in a lot of international trade, which increases the potential transfer of exotic species. Secondly, in the 1950's Elton (1958) hypothesized that low diversity levels may increase an ecosystem's susceptibility to invasion. A variety of studies has since supported this hypothesis. However, many studies have indicated that the diversity and composition of native species are not necessarily good predictors of how prone an environment is to invasion (Lockwood et al. 2013). Other factors that contribute to a more suitable environment for an invader are: climatic conditions in the newly invaded environment that match those in the native area, the absence of natural enemies, the area of invasion is anthropically disturbed, the availability of sufficient resources, and the arrangements of trophic networks (Shigesada and Kawasaki 1997; Levine and D'Antonio 1999; Holway et al. 2002; Heger and Trepl 2003).

Characteristics of the invasive organism relative to the invaded environment

The interaction between the characteristics of the invasive organism and the invaded environment plays a crucial role in the successful transition of a species from its native to a new environment. This is referred to as the key-lock approach (Heger and Trepl 2003). To add to the complexity of invasion biology, these interactions can each vary in importance depending on the invasion stage (Kolar and Lodge 2001).

The goal, in the end, is to predict the process and outcome of an invasion, which can be difficult due to the complex interplay between the various factors that influence the course of an invasion (Heger 2001). However, it can be achieved to an extent by incorporating the combined properties of the three abovementioned approaches when researching a specific invader (Heger and Trepl 2003).

Stages of invasion

The process of biological invasion consists inherently out of various ecological stages or barriers that an exotic species needs to overcome before it can inflict ecological and economic harm (Williamson 2006; Blackburn et al. 2011; Lockwood et al. 2013). Williamson and Fitter (1996) found that, on average, 10 % of species become successfully invasive. There are at least three stages of invasion, but there are different versions in terms of the number of stages that occur (Liebhold and Tobin 2008; Lockwood et al. 2013). For the purpose of this study four stages are discussed, namely: transportation, introduction, establishment, and spread (Blackburn et al. 2011).

The invasion process is initiated when a species is transported from within its native range, and released into a new area. Most species do not survive this journey (Kolar and Lodge 2001). The second stage is the introduction, which happens when a self-sustaining AIS population is created irrespective of the interacting encounters experienced in the invaded ecosystem from the time of release.

These AIS proceed to the third critical phase of the invasion process, namely the establishment. Even though the rate of world trade has increased and, as a result, the distribution of species, many founder populations, which are typically small, have failed to establish in their new environments (Liebhold and Tobin 2008; Davis 2009). During the establishment stage, population distribution grows and expands to levels where extinction is highly unlikely (Liebhold and Tobin 2008). The establishment is a function of the number of propagules (inseminated queen wasps, for example) in a dispersal (transport) event, and the number of dispersal events. The combination of these two variables is referred to as propagule pressure, which is an important driver of invasion success (Lockwood et al. 2005). The bigger the increase in the number of propagules that are imported, the higher the genetic diversity of a new population, which reduces harmful allee effects (Lockwood et al. 2005; Masciocchi and Corley 2012). The successful establishment of AIS also depends on factors associated with the new environment, such as the presence of natural enemies and mutualists, and the interaction between those factors and the AIS (Blackburn et al. 2011).

The fourth stage in the invasion process is the spread, defined as the increase in the geographic range over which the species expands over time (Arim et al. 2006). Two types of expansion occur, namely long- and short-range. The long-range expansion is a result of migratory movements, wind drift and anthropogenic movement. The short-range expansion is a result of the dispersal abilities of a species (Liebhold et al. 1995; Liebhold and Tobin 2008). The combination of short-range and long-range dispersal is known as stratified dispersal, which plays an important role in species spread (Shigesada and Kawasaki 1997; Masciocchi and Corley 2012). The spread is often not a continuous process as jump dispersal can take place, and isolated populations could eventually coalesce (Liebhold and Tobin 2008; Beggs et al. 2011; Hui et al. 2011).

Insect invasions in South Africa

South Africa has long been the recipient of deliberate and unintentional non-native species introductions (Picker and Griffiths 2011). The number of unintentional invertebrate introductions to South Africa has increased since 2000 and is expected to continue to rise (Giliomee 2011; Faulkner et

al. 2015). Although species from many taxonomic groups have invaded South Africa, impact analyses of invasive alien plants have been the subject of most studies. Therefore the impacts of invasive insects are insufficiently documented, despite being key components of native assemblages (Simberloff 1989; Williamson and Fitter 1996; Masciocchi et al. 2010) and among the most important invaders known (Richardson and Van Wilgen 2004; Van Wilgen et al. 2011; Wilson et al. 2013). For specific commercial agricultural insect pests, such as the fruit fly *Bactrocera invadens*, area-wide detection, monitoring and management programmes have been operating for the past few decades (Wilson et al. 2013).

Invasions in South Africa are unique in several respects when compared to those in other parts of the world. Due to a history of colonial occupation dating back more than 360 years, a large number of invasive alien species have been introduced (Richardson et al. 2011). Moreover, the Cape Floristic Region (CFR) in the Western Cape Province is classified as a biodiversity hotspot (Myers et al. 2000; Cowling et al. 2003). It is also one of the most heavily invaded areas in South Africa and many exotic invertebrates continue to enter the country (Geertsema 2000; Van Wilgen 2004; Giliomee 2011; Roura-Pascual et al. 2011). Therefore, the country is considered by many as the ideal environment in which to study biological invasions (Van Wilgen et al. 2014).

In general, invasive insects in South Africa, as in many other parts of the world, have had the most severe impact within human-modified systems, compared to little impact in natural systems (Mooney and Hobbs 2000). Some of the invasive insects that occur in urban areas of South Africa are cockroaches *Periplaneta americana* (L.), *Blatella germanica* (L.), the fly *Musca domestica* (L.), mosquito *Aedes albopictus* (Skuse), and the social wasps *V. germanica* and *P. dominula.* One of the exemptions is an invasive insect species, the Argentine ant, which occurs in seminatural areas of the Western Cape and has invaded fynbos (Boonzaaier 2006; Luruli 2007; Vorster 2011). Social insects are particularly effective invaders (Sackmann et al. 2008). In the case of the two invasive wasp species, their preference for human-modified systems or natural habitats within South Africa is still unknown.

Impact of alien invasive insect species

Insects form part of a highly diverse and large group of invasive organisms, but have not received the same attention given to the ecological effects caused by invasive plants, vertebrates and aquatic organisms (Kenis et al. 2009). This could be attributed to their perceived lower impact on agriculture

and the ecology (Parker et al. 1999; Beggs et al. 2011). Furthermore, studies on the ecological effects of invasive alien insects have mostly focused on specific taxa, such as ants, bees and mosquitoes; specific regions; or particular impact mechanisms. Some invaders among social insects are over-represented in studies. Eusocial Hymenoptera species, which include *V. germanica* and *P. dominula,* account for 30 % of alien insect species causing pervasive environmental impacts, according to Kenis et al. (2009), while Beggs et al. (2011) has reported they are one of the groups with the highest number of species that play critical roles in ecosystems.

AIS can directly or indirectly inflict damage on invaded communities. *V. germanica* and *P. dominula* for example, are two wasp species infamous for the threat they pose to invaded ecosystems. They are considered to be major pests to agriculture, horticulture, viticulture, biodiversity and humans (Kasper et al. 2008). Direct interactions of *V. germanica* and *P. dominula* that affect communities in their native range include: feeding on indigenous plants, preying on native animal species and, due to the scavenging nature of especially *V. germanica*, disrupting activities and stinging human beings where they are usually found in high numbers near human habitation (Clapperton et al. 1994; Sackmann et al. 2008; Tryjanowski et al. 2010; Beggs et al. 2011). *Polistes* spp. has a tendency to nest in man-made structures. Dymock et al. (1994) reported that they were the principle culprits responsible for wasp stings in Auckland, New Zealand. There are also reports by Braverman (1998) of *V. germanica* knawing on the teats of dairy cattle, causing skin inflammation and mastitis. In countries like New Zealand, where invasive wasps are one of the most widespread and densely populated invertebrate pests, predation can lead to the restructuring of native communities and the extinction of species (Beggs and Rees 1999; Masciocchi et al. 2010).

Indirect interactions between AIS and invaded communities include competition for common food sources or space with native fauna, sharing natural enemies, and carrying diseases (Tribe and Richardson 1994; Sackmann et al. 2008; Kenis et al. 2009; Pyšek and Richardson 2010). *Polistes dominula* have proven to be a highly competitive species, where characteristics such as short brood development, early production of workers and broad prey spectrum have given it an advantage over many native *Polistes* species (Cervo et al. 2000; Armstrong and Stamp 2003; Gamboa et al. 2005; Liebert 2006).

Gurevitch and Padilla (2004) argued that the presence of a biological invader is too often directly correlated to diversity loss, without being backed by truly respresentative data of the specific case study (Beggs 2001; Sackmann et al. 2008). It can be challenging and complex to obtain quantitative

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data on the impact of invasive species, but only continued research in this field can rectify the situation of information and methodology shortcomings (Kenis et al. 2009).

Global distribution of *Vespula germanica* and *Polistes dominula*

The diverse family of Vespidae wasps constitutes over 5 000 species, of which social wasps represents a fifth (Pickett and Carpenter 2010). Of the 34 vespid species that have been introduced around the world, seven of the most invasive species are eusocial, and *V. germanica* in particular has since 1945 become widespread and abundant in a variety of ecological environments (Crosland 1991; Beggs et al. 2011).

Originally from the Palearctic region (temperate Eurasia and northern Africa), *V. germanica* has been introduced to the USA, Canada, Chile, Argentina, South Africa, New Zealand, Australia and Ascension Island (Spradbery and Maywald 1992; Tribe and Richardson 1994; D'Adamo and Lozada 2009; Spradbery and Dvořák 2010; Masciocchi and Corley 2012). The common or English wasp, *Vespula vulgaris,* from its native Holarctic region, often out-compete and replace *V. germanica* in countries such as New Zealand (Matthews et al. 2000).

Another successful invasive Vespidae is of the genus *Polistes*. At least fourteen species of this genus have been found outside their supposed Palaearctic native region (Cervo et al. 2000). *P. dominula* has often become the most abundant paper wasp species in areas that it has invaded, which include most North American states and Canadian provinces, Western Australia and South America (Pickett and Wenzel 2000; Miller et al. 2013).

Distribution of *Vespula germanica* and *Polistes dominula* in South Africa

The two invasive wasps *V. germanica* and *P. dominula* were initially recorded in South Africa in Kirstenbosch and Kuils River, in 1974 and 2008 respectively (Whitehead and Prins 1975; Eardley et al. 2009). It is suspected that the first *V. germanica* specimen arrived in the Western Cape Peninsula via intercontinental transport of air cargo (Whitehead and Prins 1975; Tribe and Richardson 1994). Population expansion of *V. germanica* has been uncharacteristically slow in the Western Cape compared to other countries where dispersal rates have been documented. The wasp is still confined to a relatively small area within the Western Cape, which include on the fringes: Ceres, Wellington, Grabouw, Somerset West, Franschhoek and Constantia (Veldtman et al. 2012; Haupt 2014). *V.*

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germanica populations have been found in both undisturbed natural vegetation (Richardson et al. 1992) and in highly disturbed areas (personal observations), but it is suspected to thrive in the latter (Mooney and Hobbs 2000). Natural dispersal of queens in a favourable habitat could be 30-70 km per year (Moller et al. 1991). It is estimated that *V. germanica* spread, whether naturally, or human-aided, or by a combination of both, was 30-47 km per year in New Zealand, 60-70 km in Australia, and 64 km in Tasmania (Davidson 1987; Crosland 1991; Spradbery and Maywald 1992; Clapperton et al. 1994). During the early 2000s, the large size of excavated nests in the Somerset West area suggested that *V. germanica* overwinters in South Africa (Allsopp, pers. comm. 2014). Due to the lack of monitoring records in the timeframe between the first documented case of *V. germanica* in South Africa and the latest research conducted by Haupt (2014), it is impossible to construct successive snapshots of population spread. What can be concluded, is that *V. germanica* has spread in the past 50 years, albeit slowly, and concern remains for potential expansion beyond current distribution, where ecological factors are more favourable (Tribe and Richardson 1994). Tribe and Richardson (1994) and Spradbery and Maywald (1992) have indicated that ecoclimatic conditions for *V. germanica* are more suitable along the southern Cape coastal belt and the eastern escarpment, up toward the eastern half of sub-Saharan Africa. Once the wasp becomes established in these regions, rapid dispersal can be expected (Goodisman et al. 2001).

In the past eight years, since the first specimen of *P. dominula* was recorded in South Africa, it has spread toward Stellenbosch, Jonkershoek, Paarl, Somerset West, Strand and Grabouw (Veldtman et al. 2012; Benadé et al. 2014, Benadé 2016). Apart from the published manuscripts by Eardley et al. (2009), Giliomee (2011) and more recently Veldtman et al. (2012) and Benadé et al. (2014), noting the distribution of *P. dominula,* information regarding the population density and potential dispersal remain limited by the scope of available data and research. From personal field observations, it appears as if *P. dominula* population densities have been increasing in the past three years (2012-2015) in the Stellenbosch area.

Identification

Vespula germanica

The German wasp body is bilaterally symmetrical, with six, mostly yellow legs, two pairs of long, transparent wings and a pair of black antennae. The entire body is covered in a familiar bright black-

and-yellow colouring. Black, shield-shaped patterns run down the centre of the abdomen, with pairs of black dots to the sides of the pattern. There is a distinct, thin constriction between the thorax and abdomen (Edwards 1980). There are three tiny black spots on the clypeus (face) and the colouration pattern can vary depending on status level. Subtle differences occur between the size and body formation of males, females (sterile workers) and queens. Males are 12-18 mm long, whilst females are 12-16 mm long (Fig. 1). The thoraxes and heads are of similar size. Queens have a larger thorax and head, compared to males and workers, and are 17-20 mm long. Their abdomens are also slightly broader. Males have 13 segments in their antennae and seven visible gastral segments. The antennae of females consist of 12 segments and their abdomens have six segments with an ovipositor present on the last segment. The abdomen of the worker is shorter than that of the male and the thorax and head about the same size (Fordham 1961).

Vespula germanica constructs papery nests from chewed wood fibre that lends it a grey/brownish colour. An envelope usually encloses the tiered combs and nests are typically built below ground, although aerial nests do occur (Edwards 1980).
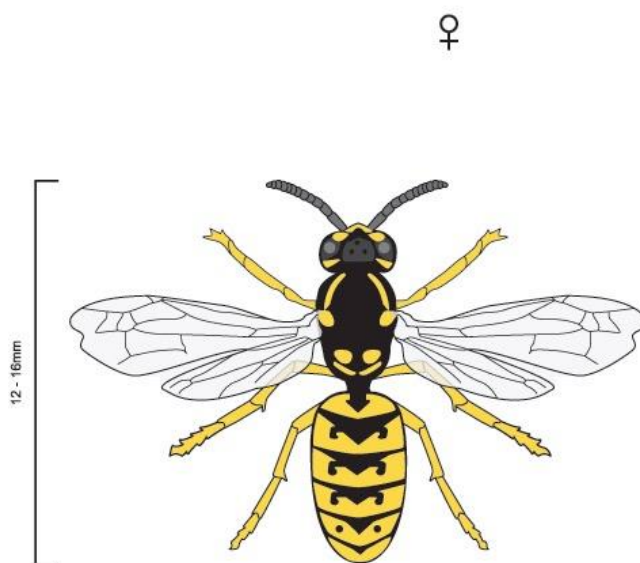
Fig. 1 A female (worker) *Vespula germanica* wasp.

Polistes dominula

The European paper wasp has a similar bright yellow-and-black colouration as *V. germanica* and is frequently mistaken for *V. germanica,* but can be distinguished by antennae of a different colour,

namely, bright yellow-orange (Fig. 2). They are known to fly with their hind legs trailing below the body. Black and yellow ringlike markings are located on the abdomen and the variation in the pattern of the three black spots on the clypeus can be associated with dominance. There is little variation among *P. dominula* individuals. On average, *P. dominula* wasps are 15-20 mm in length. The females are slightly bigger than the males and they can have longer forewings. The ventral surface of males is yellow, and black in the case of females. *P. dominula* nests are single-tiered aerial nests with the cells open and facing downwards (Dvořák and Roberts 2006; Buck et al. 2008; Cranshaw 2012).
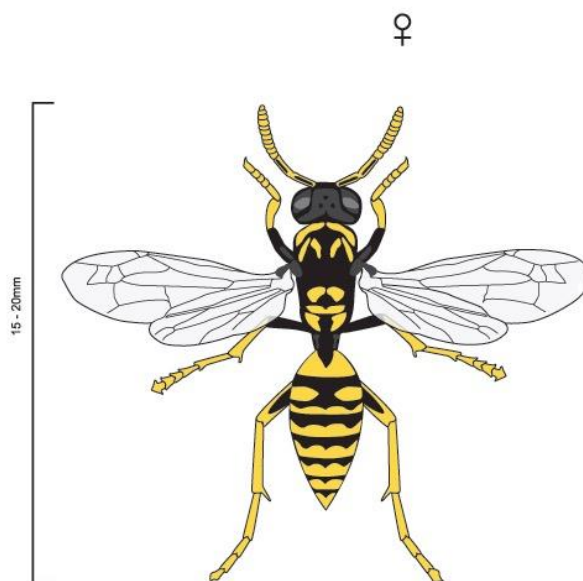
Fig. 2 A female *Polistes dominula* wasp.

Biology

In its indigenous range, *V. germanica* usually has an annual life cycle, where a single queen regulates colony activities (Edwards 1980). At the beginning of spring, a single inseminated queen initiates an embryonic nest, consisting of several hexagonal downward-facing worker cells, and starts laying eggs. Nest material consists of wooden fibres, collected by workers from trees or hedges, then mixed with wasp saliva, resulting in a product that has a papier-mâché-like consistency. The cells are enveloped in these papery layers of a nest shaped like a large golf ball. The larvae hatch after about three days. They moult three times during four to six weeks, and go through five larval instars, before pupating and developing into adult wasps, which emerge from the cocoon that covers the cell openings (Edwards 1980; Kasper et al. 2008). As soon as the first workers (females) emerge, the queen is solely devoted to

egg-laying, and the workers are responsible for expansion of the nest by adding layers of comb, defending it, foraging, feeding larvae and tending to the queen (Whitehead 1975; Spradbery & Dvořák 2010). Workers are produced throughout summer and the nest is continuously enlarged until it is about the size of a football, containing up to 10 000 cells. At that stage, colonies can consist of about 4 000 workers (Spradbery 1988). Toward the end of summer, the rate of nest expansion slows down and larger cells are built to accommodate new reproductive queens and, occasionally, males (Archer 1985). By late autumn/winter, before the queen dies and the colony disintegrates, newly produced queens leave the nest, take part in mating flights with males, are fertilised, and disperse to search for well-insulated places to hibernate (Spradbery 1973; Whitehead 1975; Kasper et al. 2008). With the advent of the following spring, hibernating queens start searching for nesting sites, and the cycle is repeated.

However, in areas with milder winters, such as Australasia, New Zealand and South Africa (Spradbery 1988; Fordham et al. 1991; Harris 1996), a small proportion of nests are able to persist for more than one year, contain multiple queens (polygynous), and grow to an enormous size (Kasper et al. 2008). These perennial colonies have been estimated to contain up to 500 000 cells per nest (Edwards 1980; Beggs 2011).

The phenology of *P. dominula* is very similar to *V. germanica* in temperate regions. However, only aerial nests are built and multi-seasonal colonies are less prevalent in invaded regions (Liebert et al. 2008; Beggs et al. 2011). *P. dominula* is also known to have two or more reproductive females sharing a nest (Höcherl and Tautz 2015).

Management

The management practices regularly used to control pestiferous agricultural insects are on the whole not effective to control social insects, due to the complex interactions they have with each other and their environment (Moller 1996). Social insects complicate both localized and large-scale management, because the caste system of social insects shields the reproductive caste from high levels of exposure to control agents that target foragers and do not significantly reduce colony size (Gentz 2009). Alternative control strategies must be applied to effectively manage and control introduced social insects (Wilson 1987).

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The first line of defence in the management of invasive organisms is to prevent them from entering a new range. Exclusion is achieved through quarantine measures. Preventing the introduction of a few individuals is normally considered to be the most cost-effective approach in the control of invasive species, but the costs associated with a comprehensive prevention plan should not be underestimated (Carlton and Ruiz 2005; Keller et al. 2008). In Cyprus and New Zealand, for example, methods were implemented to cull inseminated queen wasps before populations could become established, but it had a negligible impact on population densities, and actually in turn improved the rate of successful establishment due to a decrease in competition between inseminated queens during the following spring (Thomas 1960; Spradbery 1973). To address the impracticality and inadequacy of quarantine measures in some cases, countries such as Australia and America have imposed measures where the movement of biotic invaders is approached in an 'innocent until proven guilty' manner (Mack et al. 2000), which means that entry is allowed of any non-indigenous species that are not known to be harmful. Preventing entry of invasive species can provide an effective means to mitigate potential damage to an ecosystem. However, as soon as an invader crosses this barrier into a new environment, the focus has to be shifted to the next stage of control measures, namely eradication (Blackburn et al. 2011).

The ability to detect and monitor invader presence is an important tool necessary to manage invasive species, and to eradicate them. Surveillance techniques for insects usually involve traps containing a lure or bait. The availability of an effective lure or bait is usually the first obstacle encountered in the management of invasive wasps. Various baits and lures (chemically and physically defined) have been used worldwide to attract Vespidae, but there is no universally effective lure available for *V. germanica* or *P. dominula* that also competes economically with the range of readily available nutrient sources collected in the field by wasps (Landolt et al. 2000; Day and Jeanne 2001; Dvořák and Landolt 2006; Spradbery and Dvořák 2010; Haupt 2014).

The search for an attractive wasp lure or bait has been hampered by various factors that influence bait or lure preference. These factors include interspecific wasp species differences, geographical conspecific differences, the influence of local weather conditions on the attractiveness of lures and baits, the incidental capture of honey bees, the influence that phenological nest requirements have on bait and lure preference, and the influence of visual cues on foraging wasps (Spradbery 1973; Harris et al. 1991; Spurr 1995, 1996; D'Adamo et al. 2000, 2001, 2003, 2004; Wegner 2003; D'Adamo and Lozada 2003, 2005; Wood et al. 2006; Sackmann and Corley 2007). Once a reliable lure or bait is developed that is relevant to a specific environment and wasp species, traps can be deployed in the

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field. For monitoring Vespidae in Hawaii, it was recommended to have a minimum of five traps set 25 m apart (Rust and Su 2012).

Eradication is only feasible if the population size is small and distribution restricted. Early detection and appropriate action are vital for eradication success (Beggs et al. 2011). However, it frequently happens that ongoing monitoring is insufficient, particularly in natural areas, and as a result infestations are only detected and acted upon once they have become widespread (Crosland 1991). Moreover, detection and response time are exacerbated by a lag phase that occurs between the establishment and rapid population growth of some species (Sakai et al. 2001). Several case studies exist for both successful and unsuccessful eradication programmes. Some of these examples include the citrus blackfly, *Aleurocanthus woglumi* (Ashby), which was eradicated in the Florida Keys, the medfly, *Ceratitis capitata* (Wiedemann), from a large area in Florida, Mexico and Chile, the giant African snail, *Achatina achatina* (L.) from south Florida and Queensland in Australia, and the mosquito, *Anopheles gambiae* (Giles) from a large area in northeastern Brazil (Myers et al. 1998; Genovesi 2005; Brockerhoff et al. 2010; Pluess et al. 2012; Suckling et al. 2014). According to Wilson et al. (2013), the only documented successful eradication attempt in South Africa is that of the Mediterranean snail, *Otala punctate,* in Cape Town. On the other hand, an eradication effort that failed and even worsened the problem was an attempt to rid the southern United States of imported fire ants (Mack et al. 2000).

The window of opportunity for eradication is very small, and when it is missed, the next goal is to control the species population below an acceptable (damage) level. There are three main control approaches for well-established invaders, and they have been applied singly or in various combinations. They are mechanical, chemical and biological control, each with its own advantages and disadvantages (Spurr 1991; Barlow et al. 1996; Beggs et al. 1998; Dent 2000). Rust and Su (2012) suggest that these control measures should not be implemented until at least five Yellowjacket wasps are caught in baited traps per day.

Mechanical control

Mechanical control is the physical removal of invasive organisms and their nests by hand. It can only be effective if the geographic distribution is limited (Whitehead 1975; Edwards 1980). The method is basic, does not pose a threat to the environment, is usually well-received by the public, and has previously been highly effective. One of the methods used to successfully eradicate Giant African snails in Florida and Queensland, was to remove them by hand (Mack et al. 2000). Due to the cryptic

nature and inconspicuous nests of many social wasps, it would be extremely time-consuming to apply this method in an attempt to eradicate them, especially if nests are underground and population densities high (Whitehead 1975; Moller et al. 1991).

Chemical control

Insecticides and biopesticides remain the primary means to control insect pests and have to date been the most effective method in controlling Yellowjacket populations (Mack et al. 2000; Beggs 2001; Beggs et al. 2011). Chemicals are becoming increasingly host specific and less toxic, and are used in various reiterations to control insect pests (Menn and Hall 1999; Gentz 2009).

Trapping

Insect pests can be monitored and controlled by incorporating synthetic lures with traps. However, there are conflicting opinions on whether trapping of social wasps truly leads to a reduction in localized populations, since trapping only targets foraging wasps and not the reproductive caste (Reierson and Wagner 1975; MacDonald et al. 1976; Gangloff-Kaufmann 2002; Wegner 2003; Rust and Su 2012).

Nest treatments

Wasp nests are susceptible to a range of insecticides. If nests are not too numerous and can be easily located, they can be treated with chemicals which will kill workers and reproductives (Edwards 1980; Spurr 1993; Beggs 2011). If most nests are located in natural areas or inaccessible terrain, this method would be impractical and laborious. Even though *V. germanica* build their nests underground, they tend to nest within or near human structures, like *P. dominula,* which might make poisoning of nests within a restricted perimeter a promising option (Beggs et al. 1998; Beggs 2011).

Baiting strategies

Toxic baiting has to date been the most successful method to control a range of Vespidae species (Chang 1988; Spurr 1991; Gambino and Loope 1992; Beggs et al. 1998; Beggs and Rees 1999; Harris and Etheridge 2001; Sackmann et al. 2001; Wood et al. 2006). This method exploits the trophallactic feeding behaviour of wasps and eliminates the need to locate nests (Matsuura and Yamane 1990; Moller 1996; Harris and Etheridge 2001; Beggs et al. 2011). Workers collect attractive bait containing

slow-acting insecticide and return to the nest where they feed it to the larvae, eventually killing the brood as well as themselves (Braverman 1998; Sackmann and Corley 2007).

Many factors need to be taken into consideration with toxic baiting. Trap type, attractant/poison combination and spatial placement of traps, all play a role in the level of control obtained and impact on non-target organisms (Landolt 1998; Landolt et al. 2000, 2007; Sackmann et al. 2001).

Fresh as well as synthetic baits and lures have been used to control invasive wasp species. *V. germanica* is attracted to both protein-based and carbohydrate-based baits, whereas *P. dominula*, unlike *V. germanica*, will not scavenge off dead animals (Toft and Harris 2004; Beggs et al. 2011). The problem with fresh, sweet carbohydrate bait is that it is usually also attractive to honeybees. Therefore, the use of protein-based baits is encouraged (Edwards 1977, 1980; Dymock et al. 1991; Spurr 1996; D'Adamo and Lozada 2005; Monceau et al. 2014). However, the shelf life of fresh protein-based baits in the field is very short. This reduces their volatility and palatability to wasps (Reid and MacDonald 1986; Spurr 1995; Landolt 1998). Some studies have addressed this problem and reported that methods such as lyophilization (freeze-drying) increases storage and the shelf life of meat baits, whilst not affecting its attractiveness or toxicity (Wood et al. 2006; Sackmann and Corley 2007).

Synthetic baits have an advantage over fresh baits, because they last longer in the field, can be more host specific, elicit a stronger attraction in the target organisms, and the physical consistency makes it easy to transport (Ross et al. 1984; Spurr 1995; El-Sayed et al. 2009a, b; Lester et al. 2013; Unelius et al. 2014). Research regarding the development of chemical lures for social wasps is ongoing and has often focused on the breakdown products of fermentation (Spurr 1996; Landolt 1998; Day and Jeanne 2001; Wegner and Jordan 2005; Spradbery and Dvořák 2010). New synthetic compounds that are attractive to both *V. germanica* and *P. dominula* are continuously identified (Ross et al. 1984, Spurr 1995; Brown et al. 2013), but the most powerful species specific attractants for insects remain pheromones, which are used for pest insect control across many sectors (Witzgall et al. 2010; Brockerhoff et al. 2012; El-Sayed 2013).

Social insects rely heavily on pheromone signals to communicate and locate potential mates. By identifying the pheromones of various wasp species produced by their queens, synthetic compounds can be developed to disrupt nesting activities and mating (Brown et al. 2013; Lester et al. 2013).

In general, although baiting strategies have been efficient in reducing population densities in many cases, it achieves only localised and short-term abatement of wasp populations, because wasps from adjacent areas can invade a treated area (Beggs et al. 2011). To keep population numbers of pestiferous wasps within a small area below a certain threshold, repeated annual baiting is necessary (Whitehead 1975; Beggs et al. 1996). The efficacy of this control method also relies on the presence of a large

number of foragers, which occurs later in the season when nests are already established (Rose et al. 1999).

Alternative strategies

Aerial control of wasps through the application of baits in pellet form has been piloted by Harris and Rees (2000) in an effort to extend the area of control, but low bait–attractiveness, increased non-target risks, and potentially high costs have hampered the progress of this approach.

An understudied area of wasp management is the harnessing of molecular techniques, which could in future prove to be potentially viable. For example, RNA interference (RNAi) technology could be utilized to interfere with species-specific genes, thereby reducing non-target impacts (Ward 2013).

Disadvantages of chemical control

The potential risks to the environment and human health that accompany the use of chemicals for pest control, remain a concern (Casida and Quistad 1998). Insects develop resistance to pesticides, non-target species could be harmed, and costs escalate when repeated applications are necessary (Rose et al. 1999). There is definitely scope for improvement in the management of invasive insect species, particularly in the case of invasive wasp species, and for this reason the potential of biological control have been continuously studied (Beggs et al. 1996).

Biological control

Biological control is an intervention strategy that makes use of biotic agents to suppress a pest population (Van Driesche and Bellows 1996). Biocontrol agents such as parasitoids, pathogens, entomopathogenic nematodes (EPN), fungi, or predators, are most likely to succeed in imposing a low host equilibrium if they are host-specific, self-sustaining, have a high searching ability, their life cycles synchronize with that of the pest, and their population density can rapidly increase when the pest population increases (Murdoch et al. 1985; Lester et al. 2013).

Many organisms attack Vespidae, but the cryptic nature, colony defence mechanisms and hygienic behaviour of social wasps have yielded a poor success rate in the control of invasive wasps (Moller 1996; Gentz 2009; Beggs et al. 2011). Tribe and Richardson (1994) suggest that the limited spread of *V. germanica* over the years in South Africa might be attributed to an indigenous organism or disease. Uncovering the identity of such an agent would be significant from a biological control viewpoint

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(Whitehead 1975; Allsopp, pers. comm. 2014).

Biological control strategies can be grouped into three major categories: classical, augmentative and conservation control. Classical control entails the importation of natural enemies associated with the introduced pest species in their native range into the invaded location, reared in large numbers and released to control the pest (Hoy 2008b). Augmentative control consists of inundative and inoculative control. An inundative approach involves the release of large numbers of natural enemies for immediate control of a pest, whereas with inoculation few natural enemies are released at prescribed intervals (Hajek 2004; Hoy 2008a). Conservation control is the activity of conserving existing natural enemies that are adapted to both the environment and the pest (Barbosa 1998). The following biocontrol agents have been tested and applied in various parts of the world in attempts to control invasive wasps:

Parasitic wasps

The parasitoid *Sphecophaga vesparum vesparum* (Curtis) (Hymenoptera: Ichneumonidae), was introduced into New Zealand and Australia at around 1980 to control *Vespula* populations (Donovan and Read 1987; Donovan et al. 1989). The host specificity, short generation time and high fecundity made this parasitic wasp a promising agent (Beggs et al. 2011). The female wasp would lay eggs on the last instar *Vespula* larvae or pupae and the parasitoid larva would kill the host (Donovan 1991). However, even though the parasitoid populations established successfully at some sites, no marked effect on *Vespula* population density was observed more than twenty years after the introduction of *S. v. vesparum* (Field and Darby 1991; Beggs et al. 1996; Martin 2004; Hanna et al. 2012). This lack of control can be attributed to a moderate dispersal ability and low population increase relative to the *Vespula* species targeted (Beggs et al. 2002). Recent research by Benadé (2016) conducted in the Western Cape Province of South Africa, recorded two species of parasitoid wasps from the families *Eurytomidae* and *Eupelmidae* to emerge from both *P. dominula* and *Polistes marginales* (Fabricius) nests, which have not been recorded on *P. dominula* wasps in its native range. The exact effect that these parasitic wasps have on *P. dominula* population numbers in the Western Cape Province is not known and further research is required to determine whether it could be identified as a promising biological control candidate for future management practices.

Fungi

Entomopathogenic fungi (EPF) have been used against a range of social insects such as termites, ants and wasps, with good levels of control obtained (Kelley-Tunis et al. 1995; Rose et al. 1999). Fungal species from the genera of *Aspergillus*, *Beauveria* and *Metarhizium* have been found to be pathogenetic to social wasps (Glare et al. 1996; Austin and Hopkins 2002). Provided that the wasp forager that serves as vector for fungal spores do not die immediately, and sufficient spores are transferred to the nest, EPF shows potential as a biocontrol agent (Glare et al. 1996; Merino et al. 2007).

Nematodes

Numerous entomopathogenic nematodes (EPN) are pathogenic to social wasps. The most commonly used strains are from the genera *Heterorhabditis*, *Steinernema* and, to a lesser extent, *Mermithidae* (Poinar 1979, 1990; Lacey and Goettel 1995; Braverman 1998; Austin and Hopkins 2002). So far, EPN have in most cases been applied directly to nests (Rose et al. 1999), but they could also be infused with baits. Under certain conditions, entire wasp colonies could theoretically be killed by EPN. Additional research would further elucidate the potential of EPN as biocontrol agents of wasps (Martin 2004; Grewal et al. 2005).

Mites

Recently, a researcher discovered a mite species, (*Pneumolaelaps*) occurring in wasp nests from New Zealand. It is thought to be linked to the collapse of *V. germanica* colonies. Further research is needed to verify this hypothesis (Lester et al. 2013; Ward 2013).

Bacteria

Insect pathogenic bacteria have been found in wasp nests and their gut. Further investigation is required to determine whether these agents can be relied on to cause consistent high levels of infection (Glare et al. 1993; 1996; Austin and Hopkins 2002).

Disadvantages of biological control

One of the main concerns about biological control is unintended side effects on non-target species. Regardless of rigorous host specificity testing that is usually conducted before a biocontrol agent can be released in the field, the risk remains high, because biocontrol agents can evolve to attack other

organisms and detecting non-target impacts can be difficult (Messing and Wright 2006; Carvalheiro et al. 2008).

The special appeal of biotic agents as a management option is the possible long-term control it could provide. To achieve this, the agent must be self-sustaining to survive throughout the year until the target pest re-emerges during the following season (Rose et al. 1999). Many factors play into the survival of biocontrol agents, of which microclimate in and around the area of application is only one (Austin and Hopkins 2002). To obtain high levels of pest infection, a sufficient number or concentration of a biocontrol agent needs to be transported to the targeted colony to have a noteworthy impact. In the case of parasites, such as *Sphecophaga* spp. that attack only individual hosts, the infection levels need to be very high, which can be difficult to achieve (Martin 2004; Villemant et al. 2015).

Local regulations

Both *V. germanica* and *P. dominula* are listed as Category 1b species in the South African NEMBA regulations (National Environmental Management: Biodiversity Act 10 of 2004). According to this legislation, they are required to be controlled through an invasive species management programme (Department of Environmental Affairs 2014; Faulkner et al. 2015).

Current progress status

Tribe and Richardson (1994) suggested that efforts be made to eradicate *V. germanica* before it expanded along the southern and eastern coastal belt of South Africa. However, due to the wasps' apparent slow spread and lack of physical evidence pertaining to negative ecological, agricultural and human impacts, there has so far not been a concerted effort to eradicate it (Allsopp, pers. comm. 2014). Due to a lack/absence of records for *P. dominula* in South Africa, the deduction that this species is indeed spreading and increasing in density, can only be made from personal field observations and four published records by Eardley et al. (2009), Giliomee (2011), Veldtman et al. (2012), Benadé et al. (2014) and an online available Master's thesis by Benadé (2016). In the light of this a number of small initiatives have been launched to address the spread of both wasp species.

Firstly, multiple awareness campaigns were launched during the period of the SANBI Invasive Wasp Project (2012-2016). Newspaper articles, radio interviews and social media were used as a conduit to share and gather information.

Secondly, between 2013 and 2015 a municipal team consisting of four people was established, to follow up on telephone calls and emails regarding *V. germanica* and *P. dominula* presence in residents' gardens or public places in the Stellenbosch and Franschhoek areas (Greater Stellenbosch Municipality). During that period nests were individually removed, but a report backlog soon developed. Partly due to a lack of resources and lack of municipal interest the initiative was terminated by mid-2015.

Lastly, the Environmental Resource Management (ERM) Department's Invasive Wasp Control (IWC) Project in Cape Town formed its first team of four people in October 2014. They removed *P. dominula* nests within the Cape Town city boundaries (and two *V. germanica* nests). A second team of four was added in February of 2015. The first team responded to calls from a dedicated telephone line as well as emails. In an effort to streamline the flow of incoming reports, an online reporting tool (http://www.edrr.co.za/wasps) was implemented in 2015. In the first season 8 000 nests were removed from core areas in the northern suburbs, namely Kuils River, Kraaifontein, Durbanville, Brackenfell and Bellville. Outlier areas were Bothasig, Ottery, Wetton, Plumstead and Constantia. Altogether 1 300 nests were removed between September and December 2015 by both teams (Irlich, pers. comm. 2016). The majority had been *P. dominula* nests. A breakdown in the distribution of logged sightings for both species can be accessed at www.edrr.co.za (City of Cape Town Early Detection Rapid Response 2015). Unfortunately, a backlog formed once again. At this stage it is not possible to deal with all the respondents (Irlich, pers. comm. 2016).

Objective and aims of this study

The objective of this study was to determine the status of *V. germanica* and *P. dominula* in South Africa and the feasibility of various management strategies. During the study four experiments were undertaken and evaluated:

1. To test and determine the pathogenicity of a range of presumably indigenous biocontrol agents against *P. dominula* and *V. germanica* wasp species under laboratory conditions, with the aim to identify effective agents that could potentially be used in future to control invasive wasps in the field;

2. To determine the efficacy in the field of the EPN and EPF agents that performed best under laboratory conditions tested earlier against *P. dominula*, by means of direct spray application to nests *in situ*;

3. To use geometric morphometric techniques as a tool to provide insight into the origin of invasion and population structure of *Vespula germanica* wasps in South Africa;

4. To test the attractiveness of a range of lures and baits to *V. germanica* and *P. dominula* under laboratory conditions.

Chapters 2 to 5 are written in the style of publishable manuscripts and, for that reason, a certain amount of repetition was unavoidable.

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Chapter 2

In vivo pathogenicity of presumably indigenous entomopathogenic nematode and fungal species to South Africa, against *Vespula germanica* and *Polistes dominula* larvae

Abstract

Vespula germanica and *Polistes dominula* are known to represent a significant threat to the biodiversity of ecosystems that they invade. In South Africa, their range of distribution seems to be geographically restricted to certain regions, which make these invasive wasps good candidates for control, or eradication. The susceptibility of wasp larvae to presumably indigenous species of entomopathogenic nematodes (EPN), *Heterorhabditis bacteriophora*, *H. noenieputensis*, *Steinernema yirgalemense,* and a presumably indigenous entomopathogenic fungus (EPF), *Beauveria bassiana*, was tested. Bioassay results indicate that both *P. dominula* and *V. germanica* larvae are highly susceptible to the three EPN and one EPF screened. All larvae of both wasp species were dead and infected with EPN within 96 hours (four days) after inoculation. Similar results were obtained 168 hours (7 days) after inoculation with *B. bassiana*. Significant differences did, however, occur in the rate of infection achieved between the EPN and EPF species. Results suggest that all of the selected EPN and EPF species have the potential to be effective inundative biological control agents used within an integrated management programme for the control of *P. dominula* and *V. germanica* in South Africa.

Introduction

Since the first recordings in South Africa of the highly invasive wasp species, *Vespula germanica* (Fabricius) (Hymenoptera: Vespidae) in 1974 and *Polistes dominula* (Christ) (Hymenoptera: Vespidae) in 2008, little research, apart from bait preference trials for *V. germanica* (Haupt 2014) has been conducted on ways to control them locally (Whitehead 1975; Cooke 1985; Spradbery and Maywald 1992; Tribe and Richardson 1994; Eardley et al. 2009; Allsopp, pers. comm. 2014). Recent research indicated that populations of both species are still restricted to the Western Cape Region, where the Cape Fold Mountain Belt seems to serve as a barrier to further spread towards the north (Benadé et al.

2014; Haupt 2014). The limited distribution range creates a favourable scenario for management efforts if acted on rapidly, and increases the possibility of successful control, or even eradication of populations, if feasible (Tribe and Richardson 1994; Allsopp pers. comm. 2014).

As generalist predators and efficient competitors, these wasps have had adverse impacts on apiculture, horticulture, ecology and tourism in invaded areas around the world (Walton and Reid 1976; Edwards 1980; Akre et al. 1981; Braverman et al. 1991; Brockerhoff et al. 2010; Lester et al. 2014). Moreover, the scavenging behaviour of *V. germanica* and propensity of *P. dominula* to use manmade structures as nest-building sites, regularly bring them into close contact with humans, making them a general nuisance (Donovan 1992).

Various control methods have been developed and implemented internationally in an effort to curb population expansion of mostly the European wasp *V. germanica* and common wasp *V. vulgaris*, and to a lesser extent other social wasps (Davis et al. 1973; Edwards 1977, 1980; Moller et al. 1991a, b; Spurr 1993; Glare et al. 1996). Chemical pesticides have been adopted as a primary control measure, but have proved to achieve only temporary abatement in a localized area (Beggs 1998; Rose et al. 1999; Spurr 1991; Duthie and Lester 2013). Even though chemical control of wasps was successful in some instances (Harris and Etheridge 2001; Hanna et al. 2012; Lester et al. 2014), its use is accompanied by many disadvantages, such as non-target impacts, chemical residues, high cost of pesticides, labour-intensiveness, and impracticality when population numbers are low and terrain inaccessible (Rose et al. 1999; Sackmann et al. 2001; Merino et al. 2007). The control of social insects through fast-acting chemical pesticides presents additional challenges, due to the specific behavioural characteristics of these insects, where access to the colony and its reproductive members is protected and the intended target action of chemical agents easily impeded (Gentz 2009). Despite considerable research efforts, there is currently no panacea that ensures area-wide, long-term control of social wasps globally (Rose et al. 1999; Sackmann and Corley 2007; Ward 2013). It is therefore of great importance to improve and expand on available and new control options.

Compared to chemical control, the biological control of wasps is an under-studied area. However, it has shown potential, due to its low risk to non-target species and its self-sustaining impact over large areas (Akre 1991; Rose et al. 1999; Lester et al. 2013). Various parasitoids, predators and pathogens have been used in the past, such as parasitic wasps, beetles, entomopathogenic nematodes (EPN), and mites, to control social pests (Stimac et al. 1990; Sieberneicher et al. 1992, Pereira et al. 1993; Milner 1994; Briano et al. 1995; Beggs et al. 1996; Glare et al. 1996; Rose et al. 1999; Harris et al. 2000; Martin

2004). Significant biological control of ants, termites and wasps was achieved (Khan et al. 1993; Kelley-Tunis et al. 1995; Zoberi 1995; Glare et al. 1996; Milner and Staples 1996; Harcourt et al. 1997; Rose et al. 1999). Although attempts to control wasps using biocontrol agents have in many cases not been successful in reducing the abundance of populations (Barlow et al. 1996; Beggs et al. 1996; Beggs and Harris 2000; Beggs 2001; Donovan et al. 2002; Martin 2004; Rust and Su 2012; Lester et al. 2013), in other instances it was stated that aspects of the protocol followed could be improved to truly reflect the efficacy of the agent. Occasionally, several introductions of the control agent are required to obtain successful control (Beggs et al. 2008).

In this study, the focus was specifically on entomopathogenic fungi (EPF) and EPN, because these agents can be artificially mass-produced for inundative control applications (Ehlers 2001; Inglis et al. 2001). They can be formulated to extend shelf life and improve handling, and can be used in conjunction with other practices in an integrated pest management plan (Georgis and Kaya 1998; Shah and Pell 2003). The EPF that have specifically demonstrated potential against social wasps species such as *V. vulgaris, V. germanica* and *P. hebraeus*, are *Aspergillus flavus* (Link), a range of *Beauveria bassiana* (Balsamo-Crivelli) and *Metarhizium anisopliae* (Metchnikoff) isolates (Fenga et al. 1994; Glare et al. 1996; Rose et al. 1999; Harris et al. 2000; Lacey et al. 2001; Shah and Pell 2003; Merino et al. 2007; Brownbridge et al. 2009; Jaronski 2010). Specific EPN species that have been confirmed pathogenic against Vespidae, are *Steinernema carpocapsae* (Moller et al. 1991a, b; Gambino et al. 1992; Glare et al. 1993; 1996), *S. feltiae,* as well as Mermithid nematodes such as *Pheromermis pachysoma* (Von Linstow) and *P. vesparum* (Kaiser) (Rose et al. 1999).

The EPN infect hosts via the third, free-living, non-feeding stage, called infective juveniles (IJs). This is the mobile, virulent stage of the EPN, and the stage used in bioassay trials. IJs actively search and penetrate wasp larvae through natural openings, such as the spiracles, the anus and the mouth (Popiel and Homonick 1992). The death of susceptible hosts occurs within 48 hours (h) and is caused by the proliferation of symbiotic bacterial cells, released by the nematode into the haemolymph of the pest insect after penetration. It not only metabolises the host tissue, but also releases a substance high in antibiotics, which preserves the cadaver (Kaya and Gaugler 1993). The EPN life cycle is complete after 3-14 days and, depending on nutrient availability in the cadaver, more than one life cycle can be completed in the host. As soon as nutrients are depleted, the newly developed IJs exit the cadaver in search of a new host (Gaugler et al. 2002).

Compared to the EPN, the EPF has a slower mode of action. It also differs from the EPN in that it can directly penetrate the insect cuticle once conidia land, attach and germinate on it. No opening is

needed, and it needs not be ingested to enter the insect body (Samson et al. 1988; Roberts and Hajek 1992). Thereafter, hyphae invade the haemolymph of the host where they multiply extensively and, eventually, after 3-6 days, cause mortality. Under optimal conditions, the death of the host is followed by the emergence of the fungus through the insect's epidermal tissues after 6-7 days, after which mummification of the cadaver occurs.

The use of exotic species which co-evolved with invasive species to control pest populations in a different country, can have unpredictable and irreversible effects on the environment. Many imported biological control agents have contributed towards bio-pollution by becoming natural enemies in the introduced range (Howarth 1983; Emberson 2000; Stiling and Simberloff 2000). The establishment of exotic control agents in a new environment can lead to competition and displacement of local species, the unintended attack on non-target hosts and the dilution of the endemic faunal diversity (Howarth 1983; Emberson 2000; Messing and Wright 2006). A further complication is the absence of tests that exactly predict the outcome and effect which the release of an exotic biocontrol agent could have on an ecosystem (Messing and Wright 2006). Control agents that are indigenous, however, are climatically better adapted to local ecological conditions and should, therefore, be more efficient. Moreover, the development of natural epizootics by EPN that are indigenous is rare, because a balanced relationship already exists between natural hosts and the potential indigenous control agent (Ehlers and Hokkanen 1996; Peters 1996; Hajek et al. 2005; Jaronski 2010). To date, no naturally occurring EPF or EPN strains from South Africa have been confirmed to be pathogenic to wasps. Since social Hymenoptera clearly suffer from pathogens (Whitehead 1975; Gambino et al. 1992; Glare et al. 1996; Evison 2012; Lester et al. 2013), and invasions remain a worldwide biosecurity threat (Villemant et al. 2006; Masciocchi et al. 2010a, b; Ward 2013), further research is needed into the discovery of new entomopathogens that are presumably indigenous. The selection of effective EPN and EPF strains, under laboratory conditions, is an important step in the development of a biopesticide (Moorhouse et al. 1993; Ekesi et al. 2001; Tefera and Pringle 2004).

The aim of this research was to determine, through laboratory bioassays, the pathogenicity of three presumably indigenous EPN species and one presumably indigenous, commercially available EPF species, against larvae of *V. germanica* and *P. dominula*, and to assess the potential use of these agents as inundative biocontrol agents.

Materials and methods

Rearing of insect hosts

Galleria mellonella (Linnaeus)(Lepidoptera: Pyralidae), also called wax moth larvae (WML) were used as hosts for the *in vivo* production of infective juvenile (IJs) of EPN. Insect larvae were reared on a wheat-based diet (Table 1) in ventilated plastic containers (31.5 x 20.5 x 7 cm) at 25 °C in the dark (Van Zyl 2012).

Table 1 Ingredient composition of wax moth larvae diet*.

Ingredients	Amount per kg
Wheat flour	118 g
Wheat bran	206 g
Milk powder	118 g
Brewer's yeast	88 g
Wax powder	24 g
Honey	175 ml
Glycerol	175 ml

*Van Zyl 2012

Source of EPN

The EPN species used in the bioassay trials were all likely indigenous EPN species to South Africa from a collection at the Department of Conservation Ecology and Entomology, Stellenbosch University, which were obtained during local surveys in South Africa (Malan et al. 2006; 2011; 2014a).

All three nematode species were cultured at 25 °C according to White's (1927) amended method (Kaya and Stock 1997). IJs harvested from WML cadavers were stored in 140 ml distilled water in vented 500 ml culture flasks (Nunc[TM]) at 14 °C in the dark, aerated on a weekly basis, and used within a month after harvesting from White traps (Gaugler et al. 2000).

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Source of EPF

The *B. bassiana* fungus strain (R444) (Entomophthorales: Entomoththoraceae) that was used in the trials, is a commercially available product from Plant Health Products (Pty) Ltd., called Eco-Bb. The product is registered for use on whitefly and red spider mites and was stored in a refrigerator at 7 °C until used.

Table 2 Entomopathogenic nematodes and an entomopathogenic fungus used in trials, with their isolate number, Genbank accession number, original sample locality and habitat (Malan et al. 2006; 2011; 2014a, b).

EPN and EPF species	Isolate	Genbank accession number	Origin	Habitat
Beauveria bassiana	R444	-	Clanwilliam, Western Cape	-
Heterorhabditis bacteriophora	SF351	FJ455843	Wellington, Western Cape	Vineyard
H. noenieputensis	SF669	JN620538	Noenieput, Northern Cape	Soil under fig tree
Steinernema yirgalemense	157-C	EU625295	Nelspruit, Mpumalanga	Citrus orchard

Source of wasps

The larvae of both *P. dominula* and *V. germanica* were collected from live nests in the field in Stellenbosch, South Africa (33.9200° S, 18.8600° E), during the spring and summer (October 2012 to February 2013) and autumn in March 2013. Protective suits were worn to avoid being stung by wasps, because wasps present in and around nests were not subdued before removing the nests. In the case of *P. dominula* nests, the whole nest, and in some instances more than one nest, was removed by hand, taking care not to damage nest cells or touching the larvae. A beehive tool was used to dislodge the petiole of the nest from the structure it was attached to. *Vespula germanica* nests were generally larger than *P. dominula* nests and therefore only a few combs of a nest were used at a time, which contained enough larvae for an experimental trial to be conducted. In the case of subterranean *V. germanica* nests,

the whole nest was excavated and whole comb layers were collected. All live adult wasps were removed from the nests and combs before placing it in aerated plastic containers that were transported back to the laboratory. Larval material from both aerial and subterranean nests was used in the case of *V. germanica,* and material from aerial nests, in the case of *P. dominula*. Wasp larvae were removed from the nest cells at room temperature (25 °C), using forceps, and placed in bioassay plate wells (24 wells, flat bottom, Nunc[TM] Cat. No. 144530, Sigma-Aldrich Pty Ltd, Johannesburg, South Africa), after which they would be treated immediately.

Bioassay protocol

The *Vespula germanica* and *P. dominula* larvae were individually weighed before they were placed into filter paper-lined wells of 24-well bioassay plates. Four treatments were applied: three EPN treatments and one EPF treatment. A total of ten 24-well bioassay plates were used per treatment, of which five of those were controls, where larvae were treated with only water, which was the inoculum carrier used for application of the other treatments. Ten wells, alternately spaced on the 24-well plates, were each lined with three disks of filter paper (Whatman No. 1) with one wasp larva added per well after the EPN, EPF, or water only suspensions were respectively applied directly to the filter paper. Fungus inoculum was prepared according to the instructions on the product label. Fungal spores were applied with an Eppendorf micropipette at a concentration of 1 g spores per 1 L of distilled water, whilst the EPN were applied at a concentration of 200 IJs per insect larva. The specific dosages were selected, because the optimum dosage of the EPF and EPN isolates used against these wasps are not known. The IJ concentration has been used as base-dosage in several bioassay trials against other insect pests such as codling moth, false codling moth and citrus mealybug by the entomopathogenic nematode research team of the Department of Conservation Ecology and Entomology, Stellenbosch University, South Africa (Navon and Ascher 2000). Fungal inoculum was concentrated to the dosage prescribed by the manufacturer of the product that was used. Dose-response trials were not conducted in this study, due to a limited field supply of wasp material. The reason for inoculating only the larval stage of the wasps with biocontrol agents was that even though adult wasps in preliminary trials have shown to be susceptible to the fungus, killing only foragers does not significantly reduce wasp numbers in the field. Targeting the brood is a more practical and effective control measure (Gentz 2009).

Bioassay plates were then incubated in closed plastic containers (22 x 15.5 x 8 cm) in the dark at 25 °C and RH of > 80 %. After 24 h and 48 h respectively, the number of dead wasp larvae were recorded for

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each treatment by gently probing larvae with forceps. No movement in response to stimulation confirmed mortality.

After 48 h, filter paper disks in each well were replaced with fresh, moist disks and incubated for a further 48 h in the case of EPN treated larvae, and 120 h in the case of EPF treated larvae. In the case of the EPN treated larvae, mortality due to infection was confirmed after 96 h by means of dissection, with the aid of a dissecting microscope. The presence of IJs was visually observed. Mortality due to fungal infection was confirmed after 168 h (7 days), through visual inspection of mycosed cadavers under a dissection microscope, and visual observation of cadaver colour change to a pale pink. It was assumed that the larvae that died 48 h after inoculation with the EPN and showed positive signs of infection at 96 h after inoculation, died as a result of the EPN, because EPN kill susceptible hosts within 48 h after penetration. In the case of wasp larvae that were inoculated with EPF, it was assumed that larvae that were alive 48 h after inoculation, but infected and covered in mycelia growth 168 h after inoculation, died as a result of the EPF, since the time it takes hyphae to develop and emerge from the dead insect host is 6-7 days.

The trial was repeated on a different test date with fresh batches of entomopathogens, to ensure reproducibility of results (Butt and Goettel 2000).

Data analyses

The data were plotted against a theoretical normal to identify substantive departures from normality. Residuals were found not to be normally distributed. To determine whether there was a correlation between the mean weight of both *P. dominula* and *V. germanica* larvae and the mortality and infection levels, the nonparametric, Spearman rank correlation was used, where the null hypothesis (H_0) stated that the mean weight does not correlate significantly with mortality levels or infection levels. This analysis was conducted to determine whether larval age had an influence on the susceptibility of wasp larvae to the biocontrol agents applied. A repeated-measures ANOVA was used to compare the mortality (after 24 h and 48 h) and infection levels (after 96/168 h respectively) over time, to assess differences in the effect of the various EPN and EPF species on both *P. dominula* and *V. germanica* larvae. Additionally, significant differences between trial 1 and 2 could be compared after performing the repeated-measures ANOVA. If the residuals of the data were found to be not normally distributed, an arcsine transformation was done on the square root of the proportion dead and infected larvae at each time frame to stabilize their variances and subsequently bring them closer to normality.

Significant differences between treatment means were determined by using Fisher's LSD method. All data were analysed using STATISTICA version 12 (Statsoft Inc. 2013).

Results

Correlation between wasp larval weight and mortality and infection

Mean larval weight of both *V. germanica* and *P. dominula* species showed no significant correlation with the ability of biocontrol agents to cause mortality and infection of larvae, at any stage, from inoculation until last noted observation (Table 3). At 24 h, 48 h and 96/168 h post-inoculation, the p-values were all above 0.05 for *V. germanica*. Similar results were observed with *P. dominula*, where there was no significant correlation ($p > 0.05$) between mean weight of larvae and their mortality after 24 h, 48 h or infection after 96/168 h.

Table 3 Correlation between weight of *Vespula germanica* and *Polistes dominula* larvae and larval mortality at 24 h, 48 h and infection at 96/168 h.

Pair of variables	Species	n	Spearman	t(N-2)	p-value
Dead 24 h and weight mean	*V. germanica*	80	0.010	0.0861	0.932
Dead 48 h and weight mean	*V. germanica*	80	0.007	0.065	0.948
Infected and weight mean	*V. germanica*	80	0.082	0.725	0.471
Dead 24 h and weight mean	*P. dominula*	80	-0.128	-1.137	0.259
Dead 48 h and weight mean	*P. dominula*	80	-0.175	-1.567	0.121
Infected and weight mean	*P. dominula*	80	-0.136	-1.216	0.227

Virulence comparison of entomopathogens against V. germanica *larvae*

A significant difference ($F_{(1,70)} = 6.99$, $p = 0.01$) was observed between the first and second trial in the main effects of time and treatment, therefore the results of Trials 1 and 2 were analysed separately. The

percentage dead and infected larvae in the corresponding controls of all biocontrol treatments were below 10.5% throughout both trials. In Trial 1, 24 h after inoculation, the proportion of dead larvae treated with *S. yirgalemense* was the highest, compared to the other treatments, and the lowest proportion mortality at the same time was for *B. bassiana* treated larvae (Fig. 1). There were also significant differences between the proportion of dead larvae after 24 h, treated with respectively *S. yirgalemense*, *H. bacteriophora and H. noenieputensis*. At 48 h after inoculation, 100 % of EPN treated larvae were dead (Table 4). Infection of all of the EPN treated larvae with the three EPN species was confirmed 96 h after inoculation. After 168 h 98 % of the EPF treated larvae were infected with *B. bassiana* (Table 4).
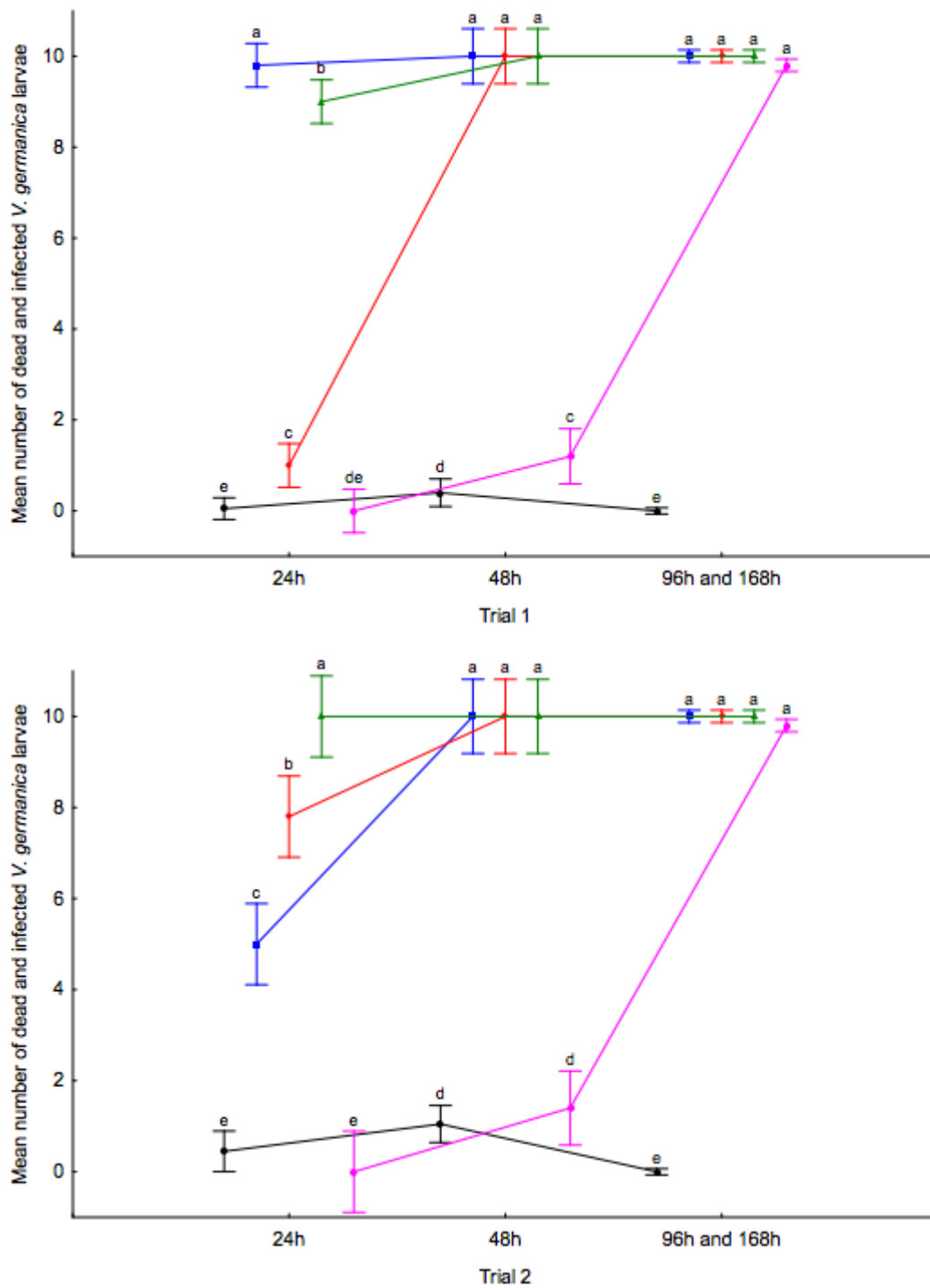
Fig. 1 The mean number of dead (after 24 h and 48 h respectively) and infected (after 96 h, or 168 h respectively) *V. germanica* larvae treated with *Steinernema yirgalemense* (■), *Heterorhabditis noenieputensis* (■), *H. bacteriophora* (■) (200 infective juveniles per insect), *Beauveria bassiana* (■) (2 x 10^9 spores per gram) and distilled water (■) (control) across time for each treatment. Repeated-measures ANOVA: Trial 1 - $F_{(8,70)} = 216.72$, $p < 0.001$; Trial 2 - $F_{(8,70)} = 76.792$, $p < 0.001$). Significant differences are indicated by different letters above 95% confidence interval whiskers.

Fig. 2 *Vespula germanica* larva infected with entomopathogenic nematodes.
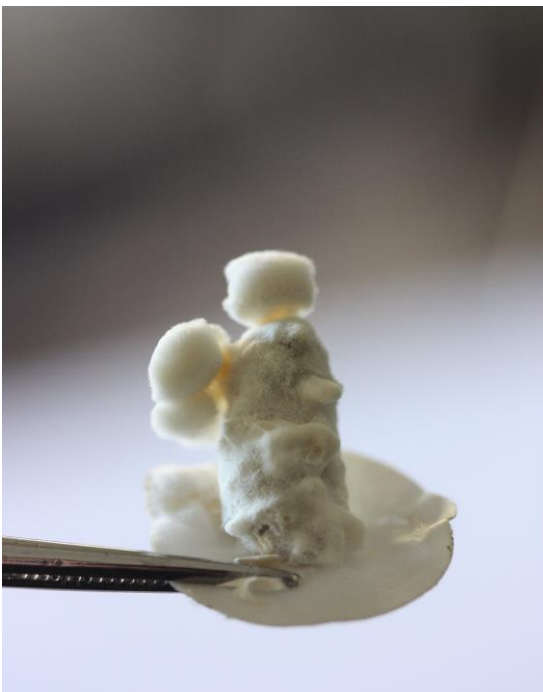
Fig. 3 *Vespula germanica* larva infected with *Beauveria bassiana.*

Table 4 Number of dead and infected *Vespula germanica* larvae (mean ± standard error) in Trial 1 for various treatments at 24 h, 48 h and 96/168 h post-inoculation. Mean ± SE followed by the same letter in a column is not significantly different at p < 0.05.

Treatment	Mean ± SE 24 h	Mean ± SE 48 h	Mean ± SE 96 h and 168
Control	0.05 ± 0.12 e	0.4 ± 0.15 d	0 ± 0.03 e
S. yirgalemense	9.8 ± 0.24 a	10.0 ± 0.3 a	10.0 ± 0.07 a
H. noenieputensis	1.0 ± 0.24 c	10.0 ± 0.3 a	10.0 ± 0.07 a
H. bacteriophora	9.0 ± 0.24 b	10.0 ± 0.3 a	10.0 ± 0.07 a
B. bassiana	0.0 ± 0.24 de	1.2 ± 0.3 c	9.8 ± 0.07 a

In Trial 2, unlike Trial 1, the larvae treated with *H. bacteriophora* showed the highest mortality after 24 h, whereas the larvae treated with *S. yirgalemense* showed the second lowest mortality (Fig. 1) if the control is not considered. The lowest proportion of dead larvae after 24 h was for the *B. bassiana* treatment. A similar trend to Trail 1 was observed 48 h after inoculation, where all of the EPN treated larvae were dead. All EPN treated larvae were also infected 96 h after inoculation (Table 5). The same number of larvae treated with the EPF as in Trial 1 (98 %), were infected with the fungus 168 h after inoculation.

The two graphs (Fig. 1) shows variability in the level of mortality caused by the three different EPN species at 24 h after inoculation in Trial 1, compared to Trial 2. However, after 48 h this variability disappeared. The EPF showed no variability between the two trails but was significantly slower than EPN in killing larvae.

Table 5 Number of dead and infected *Vespula germanica* larvae (mean ± SE) in Trial 2 for various treatments at 24 h, 48 h and 96/168 h post-inoculation. Mean ± SE followed by the same letter in a column is not significantly different at $p < 0.05$.

Treatment	Mean ± SE 24 h	Mean ± SE 48 h	Mean ± SE 96 h and 168
Control	0.45 ± 0.22 e	1.05 ± 0.2 d	-0.0 ± 0.03 e
S. yirgalemense	5.0 ± 0.44 c	10.0 ± 0.4 a	10.0 ± 0.07 a
H. noenieputensis	7.8 ± 0.44 b	10.0 ± 0.4 a	10.0 ± 0.07 a
H. bacteriophora	10.0 ± 0.44 a	10.0 ± 0.4 a	10.0 ± 0.07 a
B. bassiana	0.0 ± 0.44 e	1.4 ± 0.4 d	9.8 ± 0.07 a

Virulence comparison of entomopathogens towards P. dominula *larvae*

Significant differences were observed in the main effects of time and treatment between the first and second trial ($F_{(1,70)}$ = 19.111, $p < 0.001$), therefore the results for Trials 1 and 2 were analysed separately. The percentage larval mortality and infection obtained in the corresponding controls for each biocontrol treatment, was below 15.5% over time for both trials. In Trial 1, the treatment that caused the highest larval mortality after 24 h was *H. bacteriophora* (Fig. 4). Both *H. noenieputensis* and *B. bassiana* had the lowest larval mortality 24 h after inoculation. The three EPN treatments killed 100 % of larvae 48 h after inoculation (Table 6), and infection was confirmed after 96 h. This indicated all EPN treated larvae to be infected with the respective EPN species. Larvae treated with *B. bassiana* achieved a significantly lower larval infection (74 %) at the last recording taken 168 h after inoculation compared to the other three EPN treatments (Table 6).
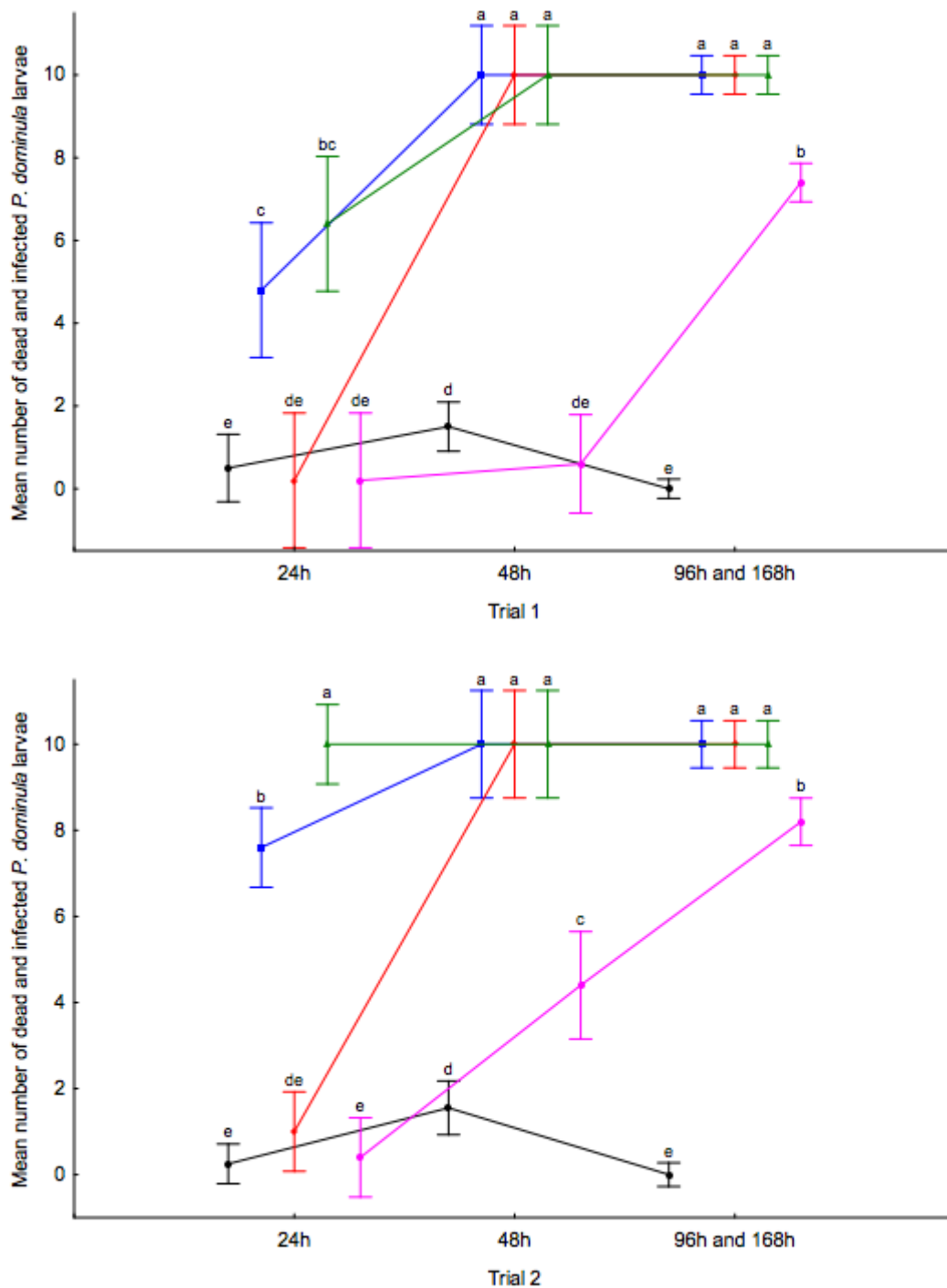
Fig. 4 The mean number of dead (after 24 h and 48 h respectively) and infected (after 96 h or 168 h respectively) *Polistes dominula* larvae treated with *Steinernema yirgalemense* (▪), *Heterorhabditis noenieputensis* (▪), *H. bacteriophora* (▪) (200 infective juveniles per insect), *Beauveria bassiana* (▪) (2 x 10^9 spores per gram) and distilled water (▪) (control), across time for each treatment. Repeated-measures ANOVA: Trial 1 - $F_{(8, 70)} = 32.748$, $p < 0.001$; Trial 2 - $F_{(8, 70)} = 31.232$, $p < 0.001$. Significant differences are indicated by different letters above 95% confidence interval whiskers.

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Table 6 Number of dead and infected *Polistes dominula* larvae (mean ± SE) in Trial 1 for various treatments at 24 h, 48 h and 96 h/168 h respectively, post-inoculation. Means ± SE followed by the same letter in a column are not significantly different at p < 0.05.

Treatment	Mean ± SE 24 h	Mean ± SE 48 h	Mean ± SE 96 h and 168
Control	0.5 ± 0.4 e	1.5 ± 0.3 d	0.0 ± 0.11 e
S. yirgalemense	4.8 ± 0.8 c	10.0 ± 0.59 a	10.0 ± 0.23 a
H. noenieputensis	0.2 ± 0.8 de	10.0 ± 0.59 a	10.0 ± 0.23 a
H. bacteriophora	6.4 ± 0.8 bc	10.0 ± 0.59 a	10.0 ± 0.23 a
B. bassiana	0.2 ± 0.8 de	0.6 ± 0.59 de	7.4 ± 0.23 b

In Trial 2 (Fig. 4), a similar trend in terms of highest larval mortality and infection achieved by each treatment through time, was observed when compared to Trial 1. At 24 h after inoculation *H. bacteriophora* killed the highest number of larvae, followed by *S. yirgalemense* and *H. noenieputensis*. All EPN treated larvae died after 48 h, while only 44 % (Table 7) larvae treated with *B. bassiana* had died after the same time. At the last recording measuring the number of larvae infected by EPN and the EPF at 96 h and 168 h, respectively, 100 % of EPN treated larvae were infected with the respective EPN species, while the EPF treatment caused 82 % larvae to die as a result of fungal infection (Table 7).

Table 7 Number of dead and infected *Polistes dominula* larvae (mean ± SE) in Trial 2 for various treatments at 24 h, 48 h and 96 h/168 h respectively, post-inoculation. Mean ± SE followed by the same letter in a column are not significantly different at $p < 0.05$.

Treatment	Mean ± SE 24 h	Mean ± SE 48 h	Mean ± SE 96 h and 168
Control	0.25 ± 0.23 e	1.55 ± 0.31 d	0.0 ± 0.14 e
S. yirgalemense	7.6 ± 0.45 b	10.0 ± 0.61 a	10.0 ± 0.27 a
H. noenieputensis	1.0 ± 0.45 de	10.0 ± 0.61 a	10.0 ± 0.27 a
H. bacteriophora	10.0 ± 0.45 a	10.0 ± 0.61 a	10.0 ± 0.27 a
B. bassiana	0.4 ± 0.45 e	4.4 ± 0.61 c	8.2 ± 0.27 b

Discussion

In this study the first investigation determined the possible influence of wasp larval weight on susceptibility to biocontrol agents. The results would give an indication of the ideal stage in the wasp life cycle or nest development at which the control agent should be applied, as wasp larvae become larger and heavier as they develop from first to fifth instar. From the results obtained, neither *P. dominula* nor *V. germanica* mean larval weight indicated any influence on the ability of the three EPN and one EPF species to infect the larvae at any stage in the experiment after inoculation. Although the p-values obtained using *P. dominula* larvae were all closer to 0.05, which might indicate a closer relationship between the mean weight of the larvae of this species to mortality and infection, compared to that of *V. germanica*, none of the p-values were below 0.05. Therefore, no significant correlation between mean larval weight and susceptibility exists for either of the two wasp species. This would suggest that all larvae in a nest that are at different stages of larval development would be equally susceptible to the biocontrol agents tested.

The second investigation of the study was to determine whether the biocontrol agents are pathogenic against the wasp larvae and, if so, which agent was the most effective. Corresponding controls were performed for each of the treatments, which served as an indicator to detect any other variable that might be influencing the results obtained, apart from the reaction in response to the applied treatment. Within 24 h and 48 h after inoculation of *V. germanica* larvae with water only, the mean percentage of

larvae that were dead for all corresponding controls, in both trials, were below 20 %. This number is negligible: according to Butt and Goettel (2000) only control mortalities higher than 20 % should be interpreted with caution. Therefore, mortality can in this case be attributed to starvation, as the larvae were not fed during the trial. Furthermore, death due to contamination can be discounted, as no contamination was observed and none of the larvae were infected with EPN or the EPF, indicating the absence of cross-contamination between the control treatments and biocontrol treatments.

In the case of the *P. dominula* trial, very low levels of mortality compared to that obtained with the biocontrol treatments were observed for the control treatments, as expected, and larval death could once again be attributed to starvation, since no contamination occurred and none of the larvae were infected with biocontrol agents.

The highest percentage of dead larvae for all corresponding controls combined in both trials was 15.5 %, presented in Trial 2, 48 h after inoculation, which was slightly higher compared to the *V. germanica* controls during the same timeframe. The higher larval mortality obtained could be an indication that *P. dominula* larvae are less resilient under stressed conditions compared to *V. germanica* larvae, where starvation might be the contributing factor. However, it would be irresponsible to infer this to be the only possible explanation, because the last time of feeding of the larvae before nests were removed from the field is unknown. The possible impact that the handling of larval material has on the results that were obtained, should also be taken into account (Butt and Goettel 2000). In one instance the control also had a higher level of mortality compared to one of the treatments. In Trial 1, at 48 h after inoculation, the mean percentage dead *P. dominula* larvae for the controls, was 15 %, as opposed to a mean of 6 % for dead fungi-treated larvae. This result can be attributed to the slow mode of infection of *B. bassiana* compared to that of EPN. The result is, therefore, not unusual. In all of the other cases, high mortality rates in fungi-inoculated larvae were observed only at a later stage after inoculation. Further support for this finding is that none of the larvae in the control were infected 168 h after inoculation, whereas 74 % of fungi-treated larvae were infected. The low level of mortality in the treatment at 48 h after inoculation is thus negligible.

All biocontrol agents used in the current study were pathogenic in varying degrees against both wasp species, but eventually resulted in high larval mortality. There were significant differences between Trial 1 and Trial 2, where *V. germanica* larvae were used, despite standardisation of the inoculation procedure. Some of the factors that could be responsible for this variation are inoculum-batch differentiation (Griffin and Downes 1994), the possible effect that the time of the last feeding can have on the susceptibility of the target insect (Kondo 1987, Flanders et al. 1996; Boff et al. 2000), host age

or larval instar (Boucias and Pendland 1998; Butt and Goettel 2000) and potential variation in the susceptibility between field populations of wasps. Little control could be exercised over these factors. Wasp samples were collected from the field, since rearing colonies under laboratory conditions can be challenging and has limitations in terms of its relevance to field conditions (Ross et al. 1981; Matthews et al. 1982; Leathwick 1997).

There were significant differences among each of the treatments in both trials. All of the EPN species applied, caused 100 % larval mortality within 48 h after inoculation, and EPN infection of all larvae were confirmed at conclusion of the trial after 96 h, making the EPN treatments equally effective in infecting *V. germanica* larvae. The noteworthy differences in the treatments were, however, observed in the rate at which mortality occurred. When comparing EPN treatments, a faster mortality rate can be an indication of a higher level of host/pathogen specificity (Brodeur 2012).

In Trial 1, *S. yirgalemense* was the fastest performing agent, killing almost all *V. germanica* larvae within 24 h after inoculation. However, in Trial 2, it was the second least effective agent after 24 h. *H. bacteriophora* caused the fastest larval mortality over both trials on average, compared to all other agents tested, and would therefore be the preferred agent to select for application in future field trials.

As expected, compared to the EPN treatments, the fungus, *B. bassiana*, performed the slowest in terms of killing larvae over time, due to a different mode of infection (Butt and Goettel 2000). Only 12 % of larvae in Trial 1 and 14 % in Trial 2 were dead 48 h after inoculation with the fungus. According to Butt and Goettel (2000), apart from host susceptibility, the speed of kill can also be related to the dose. The aqueous fungal inoculum was applied directly, at a predetermined concentration, onto the filter paper in each of the bioassay wells where the larvae were placed. Since larval movement is restricted, the probability is high that an equal number of infection propagules came into contact with the cuticle of each larva. This method of inoculation serves as a form of standardisation, but the possibility should not be discounted that a higher dosage than what is currently prescribed by the manufacturer for use on the registered insects, could cause increased levels of mortality. Future research should investigate what effect that application of varying conidial concentrations of this particular *B. bassiana* strain has on the mortality rate of *V. germanica*. In previous studies, higher spore concentrations lead to higher levels of susceptibility (Harris et al. 2000). It should, however, be mentioned that too high inoculum concentrations could lead to self-inhibition of conidia in some fungal species (Garraway and Evans 1984), which would nullify the potential benefit of increased inoculum concentrations.

At the last observation, 168 h after inoculation, the fungus was able to sporulate on the host cadaver, but levels of *V. germanica* larval infection in both trials were slightly lower compared to the larvae treated with EPN. A total of 98 % of larvae were infected with the fungus in both trials, compared to 100 % infected by the EPN species. The ability of the EPF to cause infection in *V. germanica* larvae does not significantly differ from that of the EPN species. The *B. bassiana* strain used in this study remains an attractive control option, and in some cases its delayed action can be a positive attribute, especially in the case where the success of wasp control is partly reliant on the adult wasp remaining uninhibited by the mycopesticide until it reaches the colony, inducing horizontal transmittance through the nest (Harcourt et al. 1997; Harris et al. 2000; Merino et al. 2007).

As was the case with the *V. germanica* trials, significant interaction occurred between Trial 1 and 2 of the *P. dominula* larvae, and data were analysed separately. The order in terms of the efficacy of treatments remained the same in both trials. Even though all three EPN caused mortality of all larvae after 48 h and 100 % of larvae were infected after 96 h, significant differences occurred between treatments. Once again the rate at which mortality occurred differed. Out of the three EPN species tested, *H. bacteriophora* killed larvae the fastest, with 64 % larvae dead after 24 h in Trial 1 and 100 % in Trial 2 at the same stage. The evidence that *H. bacteriophora* repeatedly outperformed the other biocontrol agents could be an indication of a good host-pathogen match.

H. noenieputensis was the least effective in killing larvae after 24 h of all the EPN agents tested, by killing only 2 % of larvae in Trial 1, and 10 % in Trial 2. It was comparable to the mortality caused by the fungus at the same stage. It seems that additional time is needed for *H. noenieputensis* to overcome the natural defences of *P. dominula*.

A similar pattern to what was observed with *V. germanica* larvae, inoculated with *B. bassiana,* was reflected in the trials for *P. dominula* larvae, where the lowest level of mortality occurred at 24 h and 48 h after inoculation. Even though the infection achieved was high at the last recording, it was lower compared to infection obtained in *V. germanica* larvae. Where a mean of 98 % *V. germanica* larvae were infected by the fungus, only 74 % (Trial 1) and 82 % (Trial 2) of *P. dominula* larvae were infected. This result suggests a slower development of *B. bassiana* on *P. dominula* larvae compared to *V. germanica* larvae and can, potentially, be attributed to the substandard activity of fungal toxins in inhibiting larval defence reactions (Harris et al. 2000; Merino et al. 2007). To summarise: *B. bassiana* showed a higher level of virulence towards *V. germanica* larvae than to *P. dominula* larvae.

According to Van Lenteren (2000), the potential of endemic biocontrol agents to control an exotic pest organism should not be overlooked, as it avoids the importation of other exotic organisms for

biological control. This study supports that statement: selected presumably indigenous EPN and EPF demonstrated high pathogenicity to two invasive wasp species under controlled conditions.

Invasions should be assessed on a case-by-case basis when the goal is to develop a tailored management programme (Shah and Pell 2003; Gentz 2009). In this case, the use of presumably indigenous EPF and EPN for wasp control should be considered. The confirmed effectiveness of these pathogenic control agents now provides the basis for future testing in the field. Future research should identify effective methods for application in the field of these pathogens. Achieving high efficacy under field conditions would be the stepping-stone towards optimising other factors such as control agent quality, its transport, storage and application (Wraight et al. 2001).

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Chapter 3

Determining the *in situ* efficacy of presumably indigenous pathogens, *Beauveria bassiana* and *Heterorhabditis bacteriophora* in infecting *Polistes dominula* larvae and pupae

Abstract

The in-field efficacy of presumably indigenous micro-organisms which are pathogenic towards *Polistes dominula* was determined. Wasp nests in the Stellenbosch and Elsenburg areas were treated from February to April of 2014, with four treatments consisting of: an aqueous solution of *Beauveria bassiana* (EPF), an aqueous solution of *Heterorhabditis bacteriophora* (EPN), a mixture of the EPF and EPN species, and a control of distilled water. The number of larvae and pupae infected 168 h after application, were calculated. In both cases the mixture of EPF/EPN performed best, with a mean percentage of 31.39 % larvae and 3.42 % pupae infected. The low levels of infection can be attributed to suboptimal conditions in the field, as well as the social structure and behaviour of wasp colonies, which protects them from pathogen invasion. The various treatments had no significant effect on the ability of wasp larvae to develop into pupae and adult wasps. In some instances temperature and humidity seemed to have influenced the percentage larvae and pupae infected. It was also found that 13 % of every nest treated was parasitized by flies, which corroborated recent published findings in a study conducted in the same geographical area by another researcher. One of the flies that emerged from a *P. dominula* nest was identified using molecular techniques. The DNA sequence matched an unclassified species from the family Tachinidae, previously found in the Gauteng province of South Africa. It is the same species that was found in *P. dominula* nests a month later by a researcher who identified it morphologically to be a fly species from the genus *Anacamptomyia*. The influence of the various treatments on fly larvae was also determined in terms of the ability of pupae to develop into adults.

Introduction

Polistes dominula (Christ) (Hymenoptera: Vespidae), often wrongly referred to as *Polistes dominulus* (Buck et al. 2008), or *Polistes gallicus*, a separate species, is a highly successful invasive wasp, native to the Mediterranean area of Europe, North Africa and temperate Asia. It now occurs on all continents except Antarctica (Carpenter 1996; Miller et al. 2013). Since its first recording in 2008 in Kuils River, South Africa (Eardley et al. 2009), the wasp has established itself in certain parts of the Western Cape, such as Stellenbosch, Jonkershoek, Paarl, the Strand, Somerset West and Grabouw (Benadé et al. 2014). Due to a generalist diet, short and productive colony cycle and often lack of natural enemies outside its native range, it spreads rapidly (Cervo et al. 2000). The ecological impact of *P. dominula* in South Africa is unknown, but in other parts of the world, they have displaced native congeners to become the dominant *Polistes* species in regions such as Canada and Northern America (Gamboa et al. 2002; Liebert et al. 2006; Beggs et al. 2011; Miller et al. 2013).

Active range expansion of *P. dominula* since its introduction seven years ago (Eardley et al. 2009; Giliomee 2011; Benadé et al. 2014) and increased abundance in the Stellenbosch area, based on personal field observations over the period of 2012 - 2014 warranted a search for agents to control the populations. Focus was directed on biological control options and identifying presumably indigenous entomopathogenic micro-organisms that are pathogenic to *P. dominula*, rather than the use of chemical pesticides. Among the many disadvantages associated with chemical control are pesticide resistance in target hosts and the health risk posed to animals and humans (Ehlers and Hokkanen 1996; Hussaini et al. 2002; Ownley et al. 2004). Several attempts at controlling invasive wasps using parasitoids have not been successful (Beggs et al. 2011). However, few studies have been conducted on means to control *P. dominula* or congener species, *in situ,* using biocontrol agents, and current research should build on the limited knowledge that does exist (Ownley et al. 2004; Paynter and Ward 2012). Moreover, searching for new biological control agents is advantageous, because an integrated pest management programme, combining chemical pesticides with biocontrol agents and cultural practices, could potentially be the most successful approach to eradicate or control invasive wasp populations (Lester et al. 2013).

Many registered, commercially available products of *Beauveria bassiana* (Balsamo-Crivelli)(Vuillemin) (Hypocreales: Cordycipitaceae) exist worldwide and much research has been conducted with regard to its biology, environmental impact and safety (Ferron 1978; Ownley et al. 2004; Tefera and Pringle 2004; Zimmerman 2007). Although *B. bassiana* is the most widely distributed

species of its genus, has an extensive host range, and is prevalent in a large number of arthropods, the majority of isolates have a restricted host range (Goettel et al. 1990; Zimmerman 2007). A range of *B. bassiana* isolates have been tested on social wasps such as *V. vulgaris, V. germanica* and *Polistes hebraeus* and has shown potential, but scant attention has been paid to pathogenicity to *P. dominula* (Paynter and Ward 2012). Moreover, there is a lack of thorough assessments of the likely impact that these specific isolates may have on non-target hosts (Rose et al. 1999; Harris et al. 2000; Merino et al. 2007; Brownbridge et al. 2009; Ward 2013). Some promise was shown in using EPN in an inundative control capacity against insect pests (Arthurs et al. 2004), but a lack of information exists concerning the use of EPN for the control of invasive wasps and its non-target impacts. Some species from the EPN genera of *Steinernema* and *Pheromermis*, have been isolated from *Vespula* wasps and confirmed as pathogens of Vespidae (Moller et al. 1991; Gambino et al. 1992; Glare et al. 1993, 1996; Rose et al. 1999; Martin 2004). However, information pertaining to the use of *Heterorhabditidae* nematodes to control *Polistes* wasps was not found.

Success in the laboratory does not necessarily translate to success in the field. The objective of this study was to determine the in-field efficacy of two presumably indigenous biological control agents that have proven to be pathogenic to larvae of *P. dominula* under controlled conditions. The two agents that performed best in bioassay tests in Chapter 2, were selected for use in this field trial, conducted in 2014 over the summer and autumn months of February, March and April.

Materials and Methods

Origin of entomopathogens

The selected EPF species, *B. bassiana*, is a commercial product, available in formulated form. It was initially isolated from soils in Clanwilliam, South Africa, by using wax moth larvae, *Galleria mellonella* L. (Lepidoptera: Pyralidae), as bait. *Heterorhabditis bacteriophora* Poinar, 1976, isolate SF351 (FJ455843), the selected EPN species, was originally isolated from soils in a vineyard in Wellington, South Africa, and the starter culture was obtained from the collection in the Department of Conservation Ecology and Entomology, Stellenbosch University (Malan et al. 2006).

EPN preparation

Infective juveniles (IJs) of *H. bacteriophora* were cultured at 25 °C according to White's (1927) amended method by Kaya and Stock (1997) using *Galleria* larvae. Emerging IJs were harvested within a week and stored horizontally in 160 ml distilled water in vented 500 ml culture flasks at 14 °C in the dark. Flasks were shaken weekly to facilitate aeration, and IJs were used in the field trial within a week after harvest. Nematodes were concentrated to 300 IJs/50 µl the day before field application using the method by Glazer and Lewis (2000).

EPF preparation

B. bassiana (R444), a strain in the commercially available product called Eco-BP from Plant Health Products (Pty) Ltd, was mixed with distilled water at a concentration of 2 g spores per 1 liter (L) of distilled water, before being applied in the field. Each gram contained 2×10^9 spores.

Field trial

P. dominula nests (n = 100) from sites in Stellenbosch and Elsenburg, were selected to be used in the field trial (Fig. 1). Temperature and humidity iButton[®] sensors (Hygrocron[®], Fairbridge Technologies, Gauteng, South Africa) were placed near nests to log ambient temperature and relative humidity (RH) every minute for 24 hours (h), from 07h00, when nests were treated, to 07h00 the following morning (Appendix A, Table 1). Medium-sized nests, with an average of 361 cells and mostly situated below roof eaves, were first sprayed with distilled water before being treated with the selected biocontrol agents. The trial took place during late summer, and autumn, (February - April), a period when *P. dominula* wasps are abundant and nests well established. Of those nests, an average of 7.75 % of the nest comprised of larvae and 11.08 %, on average, contained pupae. Samples were sprayed into vials using a hand-held spray applicator, before being sprayed onto nests. This was to confirm the presence and viability of IJs and fungal spores, after moving through the spray applicator system. With the aid of a dissecting microscope, IJs that moved either voluntarily or in response to prompting using forceps, were classified as viable specimens. The presence of fungal spores could be observed under the microscope and it was assumed that the spores were viable, since it was obtained from the same commercially available product used in Chapter 2 and applied before the expiration date stated on the product packaging. Four different treatments were applied to the respective nests, starting at 07h00 in the morning: (1) a control treatment of distilled water only; (2) *H. bacteriophora* suspension at a concentration of 300 IJs/50 µl; (3) *B. bassiana* suspension of 2 g spores/1 L; and (4) a mixture of *H.*

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bacteriophora and *B. bassiana* at the same concentrations as the separate treatments. In laboratory tests conducted beforehand to determine the pathogenicity of the specific biocontrol agents against *P. dominula* larvae, lower concentrations of *B. bassiana* (1 g spores per 1 L water) and *H. bacteriophora* (200 IJs/50 μl) were used (Chapter 2). The reason for increasing the concentrations for application in the field was to counter loss that occurs when using spraying equipment.

The respective nests were treated with the specific agent and sprayed until all cells were filled with the suspension. Nests sprayed with different treatments were not adjacent to each other, to avoid cross-contamination. After application of various treatments, nests were kept moist for a period of 6 h, by spraying it with distilled water every 2 h. After 24 h, nests were collected in separate, moist paper towel-lined plastic containers (22 x 15.5 x 8 cm), placed on overturned Petri dishes (90 mm) to avoid contact between the paper towel and the nest surface, and returned to the laboratory, where it was incubated at 25 °C, with a day/night regime of 14:10 after the number of cells, larvae and capped cells (pupae) were counted. An unexpected discovery, at that stage, was made in the process: many of the cells were parasitized with flies. Therefore, the number of parasitized cells was also recorded and included in analyses. Parasitized cells comprised, on average, 13 % of all cells in nests.

Daily readings of the number of emerging wasps and flies, newly formed wasp pupae and remaining wasp larvae were taken up to 144 h (6 days) after treatment application. Wasps were continuously removed from the containers as they emerged from capped cells and placed in alternate filter paper lined wells (moist Whatman No. 1, 15 mm circles) of bioassay plates (24 wells, flat bottom, Nunc[TM] Cat. No. 144530, Sigma-Aldrich Pty. Ltd, Johannesburg, South Africa), and incubated at 25 °C for an additional 24 h to allow for potential development of fungal growth or nematode infection. After 144 h, all larvae and pupae treated with *B. bassiana* were removed from nest cells using forceps and placed into filter paper lined bioassay wells, to be incubated for an additional 24 h at 25 °C and observed for the development of fungal growth.
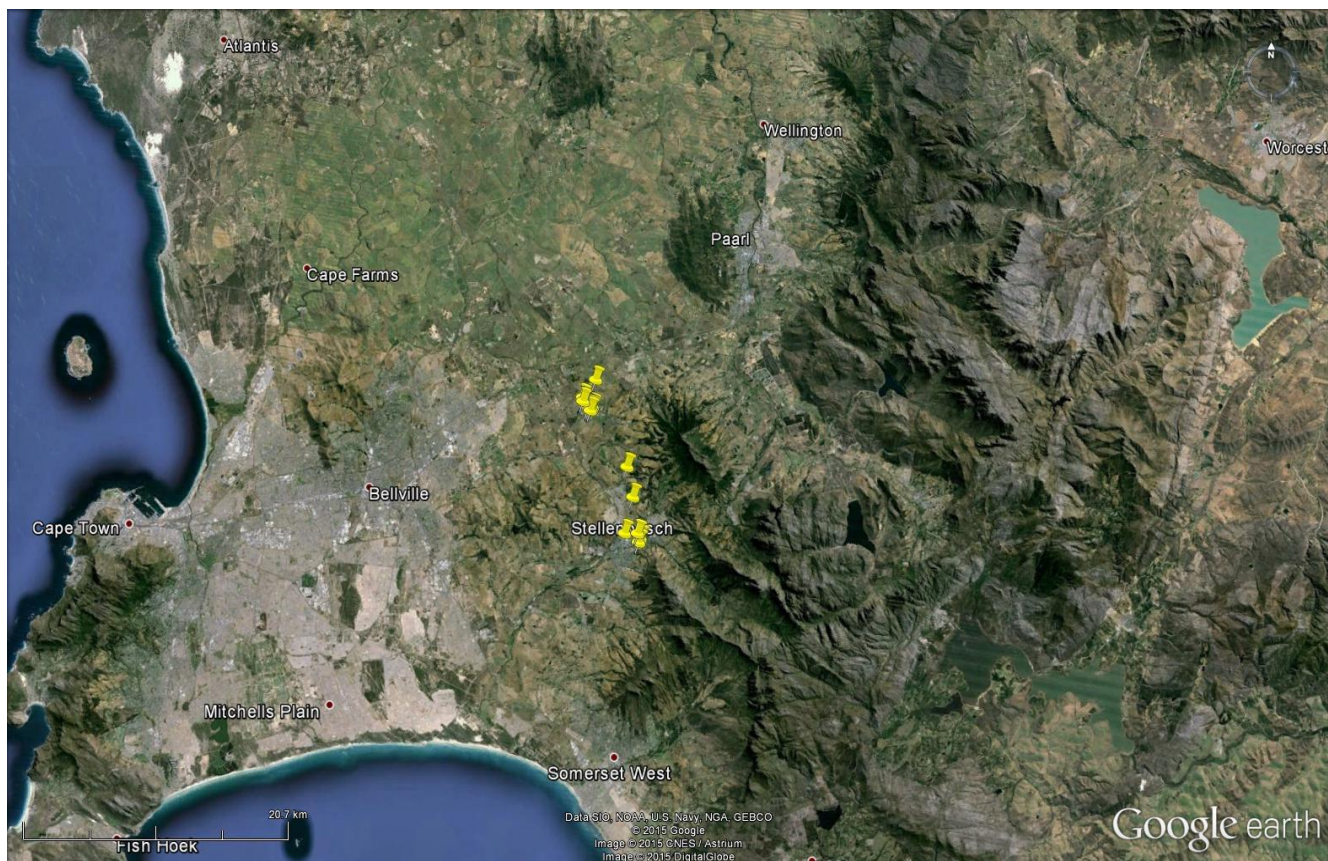
Fig. 1 Distribution in the Stellenbosch and Elsenburg areas' sites of 100 *Polistes dominula* nests which were treated with *Heterorhabditis bacteriophora*, *Beauveria bassiana*, a mixture of *H. bacteriophora* and *B. bassiana,* and distilled water, the control treatment.

Parasite

A sample of one of the flies that emerged from a *P. dominula* nest was sent to Inqaba Biotechnical Industries (Pty) Ltd, PO Box 14356, Hatfield 0028, South Africa, for molecular analyses to identify the species. The generated fly sequence was subjected to the NCBI BLAST tool as well as compared to sequences in the BOLD Identification System (IDS) for CO1.

Data analyses

Centered data were plotted against an expected normal value to test for normality. Residuals were found not to be normally distributed. A one-way ANOVA was used to compare the mean percentage infection obtained of *P. dominula* larvae and pupae, 168 h after application of the respective treatments. Post-hoc comparison of means using the bootstrap multi-comparison test was performed (Efron and

Tibshirani 1998). Linear regression scatterplots were used to determine the correlation between average temperature, average RH and percentage larvae and pupae infected.

Recordings of emerging flies over time were analysed using a repeated measures ANOVA. The residuals were not normally distributed and therefore a bootstrap multi-comparison post-hoc test was performed to determine whether treatments differed significantly from each other. All statistical analyses were performed using STATISTICA version 12 (Statsoft Inc. 2013).

Results

Susceptibility of larvae

Treatment results differed significantly from each other, $p < 0.001$; $F_{(3, 96)} = 9.256$ (Fig. 2). Nests that were treated with a mixture of *H. bacteriophora* (EPN) and *B. bassiana* (EPF), showed the highest level of larval infection and differed significantly from nests treated with only the EPN species ($p = 0.009$), or treated with only distilled water ($p < 0.001$). The control treatment, where distilled water was sprayed onto nests, showed no infection and differed significantly from all the other treatments tested. Even though the percentage larval infection obtained after being treated with *B. bassiana* was slightly higher than infection levels obtained when *H. bacteriophora* was sprayed, the treatment results did not differ significantly ($p = 0.429$).

Influence of temperature and humidity on larval infection

The only significant correlation between percentage infected larvae and average temperature, was for larvae treated with a combination of EPN and EPF ($r = 0.5$, $p = 0.02$). The higher the average temperature, the higher the percentage of larvae infected.

There was a significant correlation between average RH and percentage infected larvae for nests treated with only the EPF ($r = -0.5$, $p = 0.0063$), and with a combination of EPF and EPN ($r = 0.6$, $p = 0.0025$).
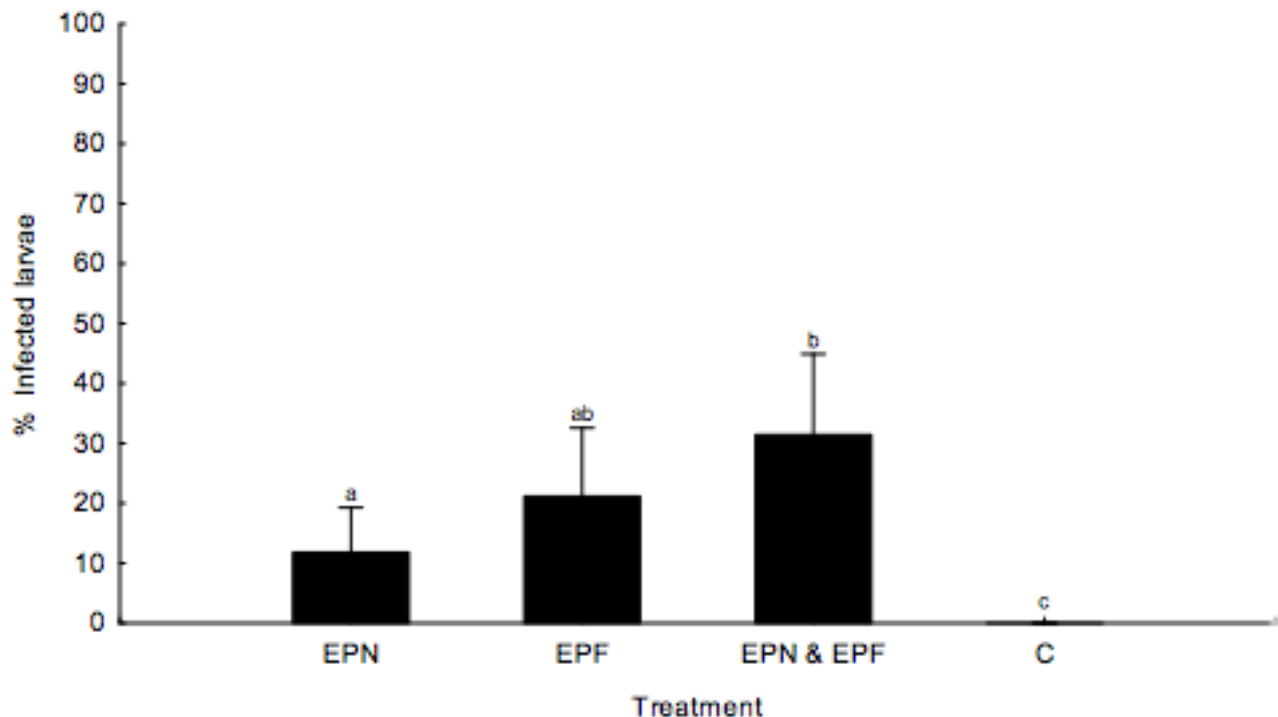
Fig. 2 The Bootstrap 95 % confidence intervals for the mean percentage infected *Polistes dominula* larvae, recorded 168 h after being treated with EPN, EPF, a combination of EPN & EPF and the control treatment (C), distilled water. Significant differences are indicated by different letters above the upper confidence limit.

Susceptibility of pupae

In all of the treatments, the percentage pupae infected with control agents were much lower compared to larvae infected in nests. A similar trend, compared to larval infection, was observed in terms of the efficacy of each of the treatments (Fig. 3). The highest mean percentage of infected pupae was obtained in nests treated with a combination of EPN and EPF.

There were significant differences between treatments and pupal infection obtained ($F_{(3, 93)}$ = 5.0, p = 0.003). The joint treatment of EPN and EPF differed significantly from the control treatment (p = 0.009). There were no significant differences between the other treatments (Fig. 3).

Influence of temperature and humidity on pupal infection

There was a correlation between the percentage infected pupae treated with a combination of EPN and EPF, and the average temperature (r = 0.5, p = 0.02). The mean percentage infected pupae increased as the average temperature increased. No correlation was observed in any of the other treatments. Neither

was there a correlation in any of the treatments between the percentages infected pupae and the average RH.
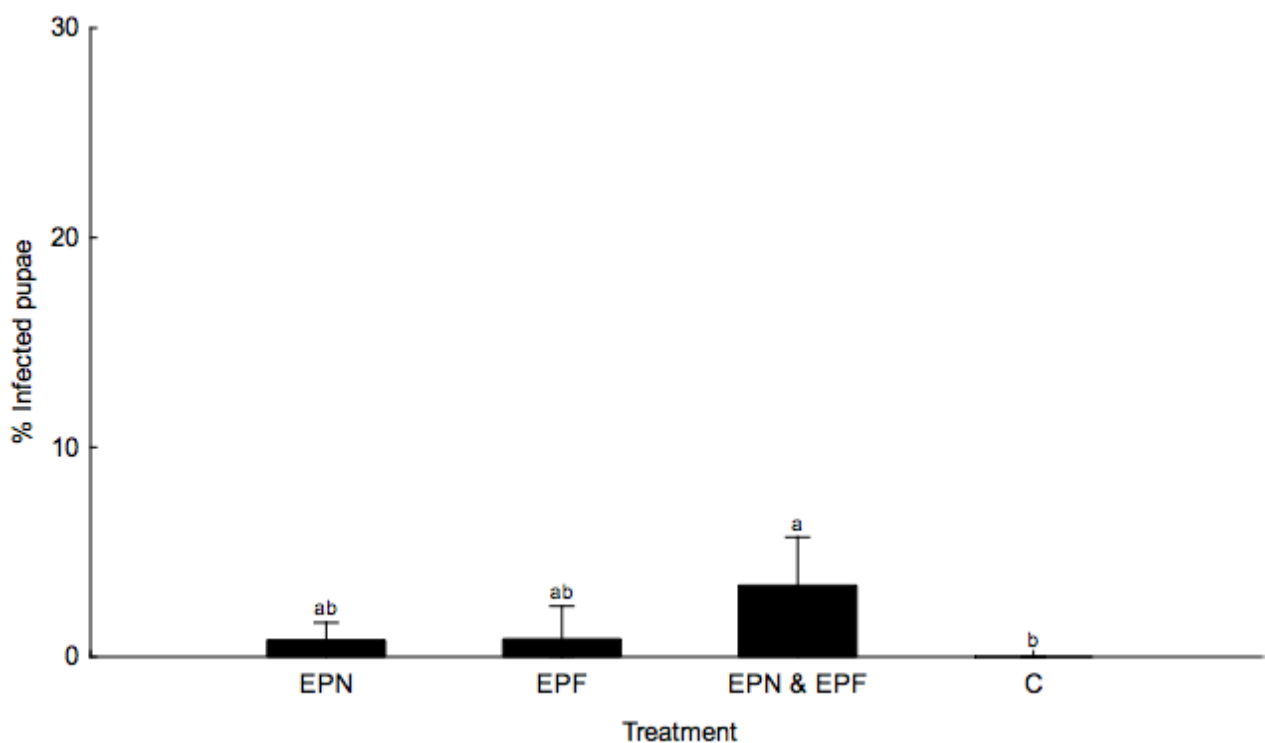
Fig. 3 The Bootstrap 95 % confidence intervals for the mean percentage infected *Polistes dominula* pupae recorded 168 h after treatment with EPN, EPF, a combination of EPN & EPF and the control treatment (C), distilled water. Significant differences are indicated by different letters above the upper confidence limit.

Polistes dominula *larvae that developed into pupae.*

No significant differences (Fig. 4) were observed in the percentage larvae that developed into pupae after the various treatments were applied ($F_{(3, 93)} = 0.09712$, p = 0.96144).

Effect of temperature and humidity on larvae that developed into pupae

The only correlation between the average temperature and mean percentage larvae that developed into pupae, was for nests treated with EPN (r = 0.4, p = 0.03). As the temperature rose, the number of pupae formed increased.

A significant correlation between larvae that developed into pupae and the average RH was observed for nests treated with distilled water ($r = -0.67$, $p < 0.001$), EPN ($r = -0.5$, $p = 0.009$) and EPF ($r = -0.51$, $p = 0.01$), respectively. With these treatments, a decrease in the RH was correlated with an increase in the mean percentage of larvae that developed into pupae.
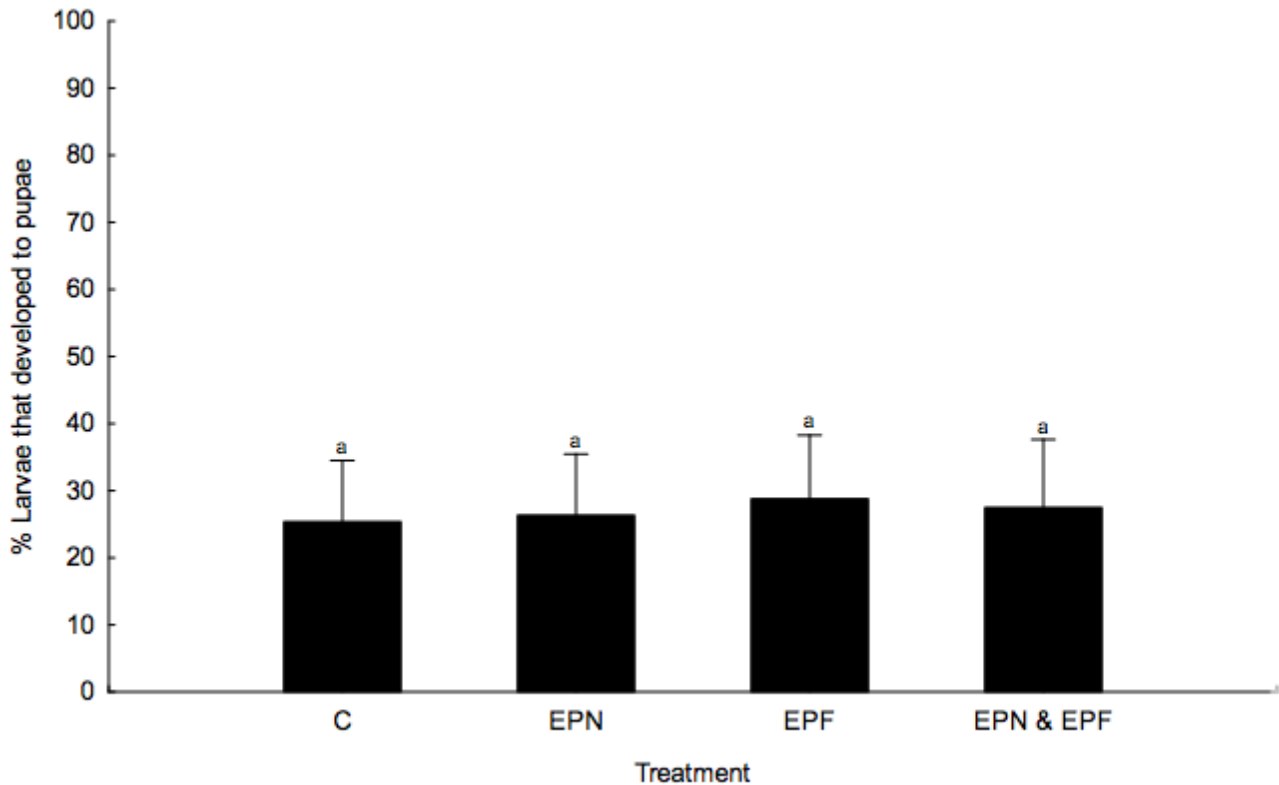
Fig. 4 The Bootstrap 95 % confidence intervals for the mean percentage of *Polistes dominula* larvae, recorded over a period of 144 h, that developed into pupae after being treated with EPN, EPF, a combination of EPN & EPF, and distilled water (C), respectively. Significant differences are indicated by different letters above the upper confidence limit.

Polistes dominula *pupae that developed into adult wasps*

No significant differences (Fig. 5) were observed in the percentage of pupae that developed into adult wasps after the various treatments were applied ($F_{(3, 93)} = 0.86157$, $p = 0.46400$).

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Effect of temperature and humidity on pupae that developed into adult wasps

There were no significant correlations between the average temperature or RH and the percentage of pupae that developed into adult wasps, for any of the treatments.
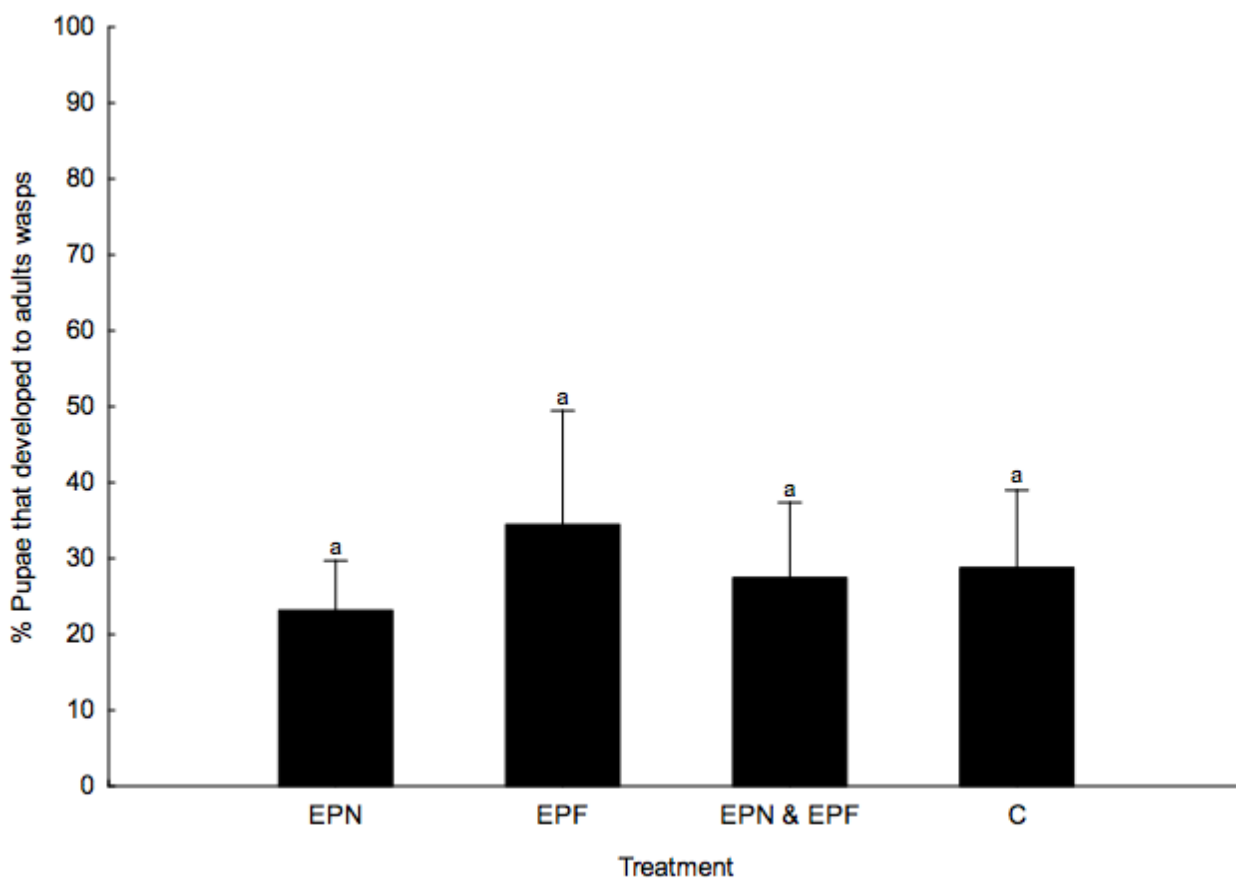
Fig. 5 The Bootstrap 95 % confidence intervals for the total mean percentage *Polistes dominula* pupae, recorded over a period of 144 h, that developed into adult wasps after being treated with EPN, EPF, a combination of EPN & EPF and distilled water (C). Significant differences are indicated by different letters above the upper confidence limit.

Fly parasitism

A 100 % match with an uploaded sequence (Sequence ID: GMSAB2603-13) from the BOLD Identification System (IDS) database was found. It matched that of an unclassified fly species (Fig. 6) that was collected in the Gauteng Province of South Africa in 2012.

Fig. 6 An unclassified fly species from the Tachinidae family that was collected from a *P. dominula* nest in Stellenbosch, South Africa. (Specimen image: BOLDSystems).

Percentage fly pupae that developed into adult flies

No significant differences (Fig. 7) were observed in the percentage fly pupae (parasitized cells) that developed into adult flies after the application of the various treatments and the control ($F_{(3, 93)} = 2.70$; $p = 0.5$).

Effect of temperature and humidity on fly pupae that developed into adult flies

There were no significant correlations, at any stage after application of the various treatments, between the percentage of fly pupae that developed into adult flies, and the average temperature and RH.
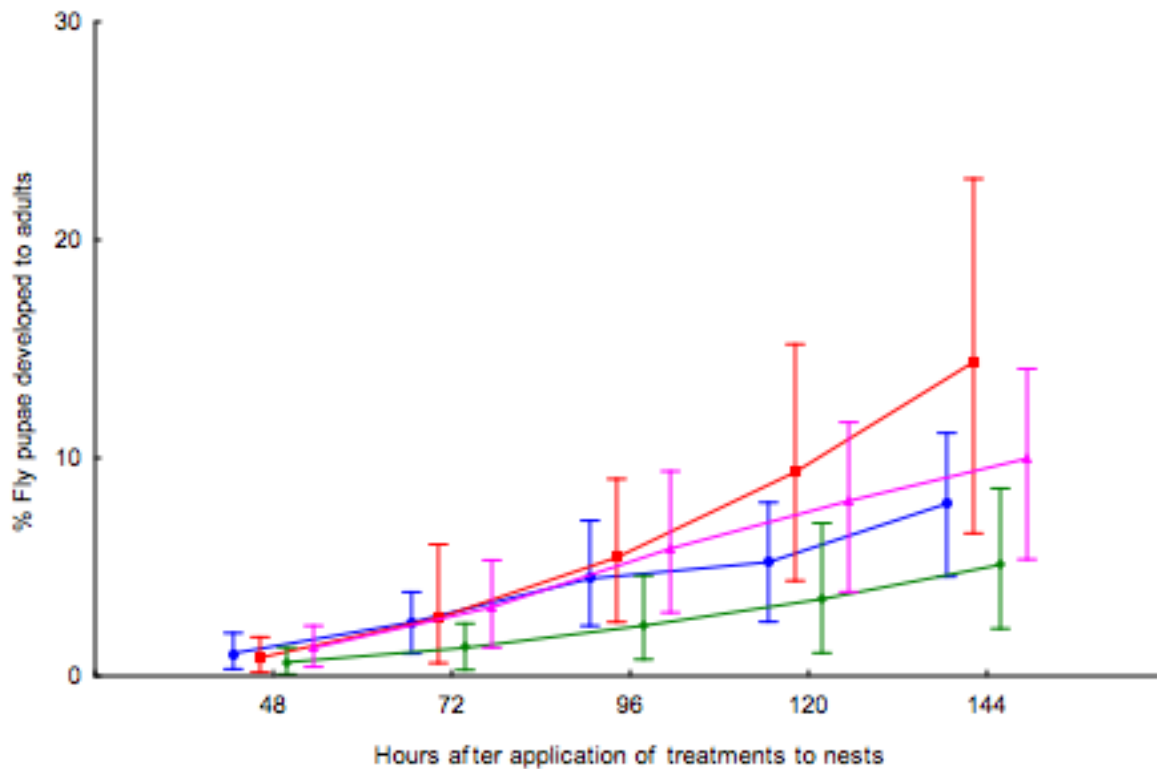
Fig. 7 The Bootstrap 95 % confidence intervals for the mean percentage of fly pupae that developed into adult flies, recorded over a period of 144 h after being treated with EPN (■), EPF (■) , a combination of EPN & EPF (■), and the control, distilled water (■).

Discussion

The promising results obtained in Chapter 2, where high levels of infection were achieved against both *V. germanica* and *P. dominula* larvae, using presumably indigenous *B. bassiana* and *H. bacteriophora* species, motivated the decision to test these biocontrol agents under field conditions, which is often sub-optimal in terms of humidity and temperature. All of the biocontrol agents were applied as an aqueous solution and no additional substances, such as spreaders, stickers or humectants were added, because the focus of this study was to determine the efficacy of control agents, and not to improve the formulation. Moreover, application guidelines of the commercial product *B. bassiana* state that no adjuvants should be added. Uniformity was ensured by applying the same guidelines to the other treatments to enable later independent comparisons.

The mean percentage *P. dominula* larvae that were infected at conclusion of the experiment, was much lower for both EPF and EPN treatments, respectively, compared to the results obtained under

controlled conditions in the laboratory. Under optimal conditions in the laboratory, 74 %/82 % and 100 % of *P. dominula* larvae were infected 96 h after application of *B. bassiana* and *H. bacteriophora*, respectively (Chapter 2). Only 31 % of larval infection was obtained when a combination of these EPN and EPF species were applied to nests in the field. Many abiotic and biotic factors could be responsible for the result.

Firstly, abiotic constraints such as temperature, humidity and UV exposure, have a notable effect on the ability of EPN and EPF to infect a host. High humidity levels (>70 % RH) are required for at least 8 – 24 h after the application of agents such as EPN and EPF, to ensure successful germination and infection (Georgis and Gaugler 1991; Arthurs et al. 2004; Zimmerman 2007; De Waal et al. 2013). The RH in the field ranged for all sites between 46 % and 73 % during the time from application until nests were removed 24 h later. In an effort to keep nests moist, they were sprayed with distilled water before the treatments were applied, and up to 6 h after application (Womersley 1990a, b), but even though micro-humidity in nests were high enough to support EPN and EPF infection, which occurs within 24 h (Jaronski 2010), very high ambient temperatures were experienced on certain days. The average temperature was between 18 – 31 °C during the trial period, but a high of 58 °C was once logged on a day in late February (Appendix 1, Table 1). Regardless of the high temperature, the EPF and EPN were, surprisingly, still able to infect larvae. The optimum temperature for *B. bassiana* is between 20 – 30 °C (Roberts and Campbell 1977; Tefera and Pringle 2003) and at temperatures higher than 50 °C the fungus would most definitely be killed (Walstad et al. 1970). The optimal temperature range for *H. bacteriophora* to infect insect hosts is between 1 – 32 °C and the EPN would also be rendered inactive or die at such high temperatures (Grewal et al. 1994). In an effort to minimize the influence of high temperatures on biocontrol agent efficacy, agents were applied early in the morning, but during the course of the day temperatures increased and due to *P. dominula's* propensity to build nests on the eastern side of manmade structures above ground, morning sun raises the temperature and exposure to extreme thermal conditions could not be avoided entirely. Solar radiation also affects the field persistence of fungal insecticides (Zimmerman 2007). In laboratory trials conducted by Fargues et al. (1996), most *B. bassiana* isolates were killed within 2 h after exposure to UVC, UVB and UVA rays. EPN from the *Heterorhabditis* genus are particularly UV intolerable. Studies by Gaugler et al. (1992), showed rapid inactivation of *H. bacteriophora* IJs at exposure to medium UV (302 nm).

Secondly, biotic constraints, such as the behavioural characteristics of social wasps, further impede the ability of biocontrol agents to infect progeny. Wasps actively suppress growth and dispersal of

biocontrol agents in nests, by removing diseased nest mates and, in addition, the presence of antimicrobial compounds in wasp saliva, imbedded in cell walls of nests, inhibits fungal development (Rose et al. 1999). *P. dominula* nests are not enclosed in a papery layer like *V. germanica* nests. This results in the easy removal of infected material and lowers persistence of biocontrol agents in nests.

Even though levels of larval infection obtained were low, there were significant differences between the efficacies of the treatments. The treatment consisting of both EPN and EPF performed best and was the only treatment that differed significantly from the rest. Several studies have reported observing a synergistic effect when EPN, EPF and chemical pesticides were used in combination with each other to combat insect pests (Barbercheck and Kaya 1990; Ericsson et al. 2007; Ansari et al. 2010). In a study conducted by Shairra and Noah (2014), treating the cotton leaf worm (*Spodoptera littoralis*) (Lepidoptera: Noctuidae) with combinations of EPF species, *B. bassiana* and *Metarhizium anisopliae,* and EPN species, *H. bacteriophora* and *S. riborave*, increased mortality levels in most combinations, compared to when each pathogen was applied individually.

As expected, infection levels of wasp pupae were much lower compared to that achieved for the larvae, because pupae in *P. dominula* nest cells are covered with a silk-like cocoon formation. The best performing treatment was the EPF and EPN combination, but an average of only 3.42 % pupae were infected, which is a tenth of the percentage of larvae that were infected using the same treatment. From this outcome it is clear that the cocoon formation, which encapsulates the pupa, serves as a filter by impeding entry of biocontrol agents and creating the opportunity for pupae to successfully develop into adult wasps. No significant differences between the control, and any of the other treatments were obtained in terms of the percentage pupae that eventually developed into adult wasps over time. Some of the larvae that were sprayed, were not infected and developed into pupae, regardless of which treatment was applied. No significant differences in the percentage of larvae that developed into pupae were observed, among treatments. Older larvae of insects tend to be less vulnerable to infection, due to reduced food intake and a thicker cuticle, which could make pathogen penetration difficult (Teakle et al. 1986; Kondo 1987; Purwar and Sashan 2005; Bukhari et al. 2010; Perry et al. 2013). However, results obtained in Chapter 2 do not reflect these findings with regards to *V. germanica* and *P. dominula* larvae. It was found that the weight of both *P. dominula* and *V. germanica* larvae, respectively, had no significant influence on the susceptibility of the larvae to biocontrol agents tested. Therefore, in this case, larval age could not have been a contributing factor in the ability of some of the larvae to develop into pupae.

In the process of removing *P. dominula* nests after being sprayed, it was noticed that many cells in each of the nests were parasitized. On average, 13 % of every individual nest sprayed, was parasitized with fly pupae. This result is in accordance with a study conducted by Benadé (2016), where parasitic flies from the genus *Anacamptomyia* (Tachinidae) emerged from nests of both the invasive wasp *P. dominula* and the native wasp, *Polistes marginales* (Fabricius). The number of flies that emerged from all of the treated nests over an incubation period of 144 h, was then recorded. Flies emerged at an increased rate over the observed period. There were no significant differences between the percentages of flies that developed into adults that originated from nests treated with biocontrol agents versus nests treated with the control. It might be a possibility that this specific fly species is not susceptible to the biocontrol agents tested. However, the remaining fly pupae were not investigated for fungal or nematode infection and therefore future research should aim to determine the pathogenicity of these biocontrol agents against the specific fly species to confirm or reject this hypothesis. This information could be useful in terms of expanding current knowledge of the host range of the presumably indigenous *H. bacteriophora* and *B. bassiana* species, as well as being another factor that could be beneficial to take into consideration when developing an integrated pest management programme. In selecting biocontrol agents that will not kill fly larvae and pupae, the additive effect that their presence in wasp nests might have on reducing wasp larvae numbers, could be noteworthy (Toft et al. 1999).

Several parasitoids have been associated with *P. dominula* in both its native and invaded range, which include *Endurus argiolus* (Rossi) (Hymenoptera: Ichneumonidae), *Xenos vesparum* (Rossi) (Strepsiptera: Stylopidae), *Polistes sulcifer* (Zimmermann); *Dibrachys cavus* (Walker) (Hymenoptera: Pteromalidae), *Chalcoela iphitalis* (Walker) (Lepidoptera: Pyralidae) and *Sacophaga* sp. (Diptera: Sarcophagidae) (Dapporto et al. 2004; Beani et al. 2011; Miller et al. 2013). No research, apart from the study by Benadé et al. (2014), has been conducted in determining the parasitic composition of *P. dominula* in South Africa. Three parasitoid taxa were recorded to emerge from *P. dominula* nests. One included a fly from the genus *Anacamptomyia* sp. (*Tachinidae*) (Benadé et al. 2014). The fly specimen was identified by means of morphological techniques and is the same species as the one identified in this study. Six Diptera species are known to attack *Polistes* wasps (Paynter and Ward 2012) and there are reports of flies having a serious impact on *Polistes* colonies in Colombia (Yamane 1996) and conopid flies (*Physocephala tibialis*) on bumblebees (Villemant et al. 2015). However, the exact effects of these parasitic flies on *P. dominula* colonies in South Africa are unknown.

In this study, low infection levels of both larvae and pupae were obtained in treating *P. dominula* nests with presumably indigenous *H. bacteriophora* and *B. bassiana* species. Therefore, several aspects of the in-field use of these EPN and EPF agents should first be improved to increase their efficacy. Selecting heat tolerant EPN/EPF strains and more suitable application equipment can substantially influence the impact on pathogenicity levels (Shapiro-Ilan et al. 2006). Proteins present in wasp saliva result in hydrophobic-produced paper of which nests are built (Espelie and Himmelsbach 1990), and can be responsible for poor adhesion of biocontrol agents to nests, especially if the agents are applied in an aqueous form. To increase coverage and limit desiccation, adjuvants can be incorporated into biocontrol formulations to increase humidity, and sedimentation of organisms in aqueous solutions can be reduced (Van Niekerk 2012; De Waal et al. 2013). By introducing UV protectants to formulations, the extreme sensitivity of both EPN and EPF to solar radiation can be lowered (Wraight et al. 2001). Should control agents be sprayed earlier in the season (September - October), wasp nests of single/two queen colonies will be smaller and attack from control agents harder to fight.

It is clear from the results obtained, that the highest efficacy of the selected biocontrol agents would be achieved by applying it to nests when nest cells contain mainly larvae, since larvae were overall more susceptible to the biocontrol agents tested, than pupae. It would, therefore, be preferred to apply the best performing biocontrol agent, which was the combination of EPN and EPF, to wasp nests at the beginning of the wasp season, when nests are still small and mostly filled with larvae and not pupae.

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Appendix A

Table 1 Minimum, average and maximum temperatures/relative humidity at treatment sites during field applications of biocontrol agents.

Date	Location	Temperature °C			Humidity % RH		
		Min	Average	Max	Min	Average	Max
27/02/2014	33°50'48.82"S, 18°49'02.87"E	17.6	26.6	40.1	19.3	54.1	91.3
27/02/2014	33°50'38.81"S, 18°49'1.35"E	17.6	26.6	40.1	19.3	54.1	91.3
27/02/2014	33°51'01.62"S, 18°49'20.41"E	17.6	26.6	40.1	19.3	54.1	91.3
27/02/2014	33°54'52.12"S, 18°51'43.06"E	19.1	31.4	58.0	11.0	46.2	76.4
27/02/2014	33°49'52.89"S, 18°49'42.65"E	18.6	29.2	46.1	14.3	47.9	83.1
27/02/2014	33°56'22.90"S, 18°51'17.61"E	17.9	26.5	34.9	30.9	52.7	84.3
6/03/2014	33°51'09.13"S, 18°49'29.80"E	18.6	25.8	37.1	33.2	63.3	91.2
6/03/2014	33°51'07.06"S, 18°49'26.11"E	17.1	24.6	36.6	35.6	69.5	95.2
6/03/2014	33°51'5.13"S, 18°49'19.49"E	17.1	24.6	36.6	35.6	69.5	95.2

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13/03/2014	33°51'02.39"S, 18°49'34.60"E	13.1	22.6	33.1	16.3	47.4	81.8
20/03/2014	33°53'35.38"S, 18°51'16.70"E	10.1	20.8	36.1	31.3	65.1	99.6
27/03/2014	33°51'07.06"S, 18°49'26.11"E	15.6	22.7	33.6	40.4	72.7	93.3
27/03/2014	33°56'34.83"S, 18°52'06.74"E	16.1	24.0	37.6	32.2	66.8	94.6
27/03/2014	33°56'23.91"S, 18°51'59.40"E	16.5	24.2	46.0	32.2	66.8	94.6
27/03/2014	33°51'09.13"S, 18°49'29.80"E	15.6	22.7	33.6	40.4	72.7	93.3
27/03/2014	33°51'5.13"S, 18°49'19.49"E	15.6	22.7	33.6	40.4	72.7	93.3
27/03/2014	33°51'01.62"S, 18°49'20.41"E	15.0	22.2	33.0	40.4	72.7	93.3
27/03/2014	33°51'12.88"S, 18°49'26.62"E	15.6	22.7	33.6	40.4	72.7	93.3
3/04/2014	33°51'07.06"S, 18°49'26.11"E	12.1	18.4	28.6	41.1	63.4	90.7
3/04/2014	33°51'09.13"S, 18°49'29.80"E	12.1	18.4	28.6	41.1	63.4	90.7
3/04/2014	33°56'46.43"S, 18°52'01.82"E	13.1	18.7	28.6	40.1	59.9	85.7
10/04/2014	33°56'46.43"S, 18°52'01.82"E	22.6	26.1	29.6	47.8	52.2	55.4

Chapter 4

Origin and invasion pathway of *Vespula germanica* in South Africa, as determined by variation in wing shape using geometric morphometric analyses as differentiation tool

Abstract

Landmark–based geometric morphometric analyses were used to investigate the variation in forewing shape of *Vespula germanica* worker wasps from widely separated geographic locations around the world to gain information about the geographic origin of South African *V. germanica* populations, as well as to identify the possible invasion route that was followed within South Africa. A total of 845 wasps were collected over two years from five different geographic areas: France (native), and four invaded parts including South Africa, Argentina, Australia and New Zealand. Significant variation in the wing shape of wasps from the thirteen collection sites was present, and shape differences in relation to the distance that each of the groups were from each other in a tangent shape space, were evident. The analyses inferred that the source of invasion most likely stemmed from Europe and that wasps had spread from Kirstenbosch via Somerset West, through Stellenbosch, to Franschhoek. A direct correlation between wing shape and geographic distance was observed, which suggests that morphological differentiation in South African *V. germanica* populations can be explained as isolation-by-distance.

Introduction

Vespula germanica is a highly invasive wasp species that has a strong capacity to adapt to environments which vary in climate and topography and it has severe environmental, agricultural and human impacts (Kasper et al. 2008; Beggs et al. 2011). Due to its high plasticity the wasp has spread quickly and reached high densities in invaded regions. It now occurs on all continents, except Antarctica (Crosland 1991; Beggs et al. 2011). One of the regions invaded by *V. germanica* is the Cape Floristic region (CFR) in the Western Cape Province of South Africa, a biodiversity hotspot known to have been highly invaded by accidental introductions of both fauna and flora (Cowling et al. 2003;

Roura-Pascual et al. 2011). The first specimen was found in 1974 in Kirstenbosch, even though the wasp might have been present before that (Whitehead 1975; Whitehead and Prins 1975). Even though the CFR has shown to be a marginally favourable habitat for *V. germanica* (Tribe and Richardson 1994), the wasp has managed to spread, albeit slowly compared to other invaded countries like Australia and New Zealand, and now occurs in certain parts of the Western Cape Province (Haupt 2014).

Indigenous to Europe, North Africa and temperate Asia, the first specimen of *V. germanica* found in the United States was from a museum collection dated 1891 and collected from Ithaca (Menke and Snelling 1975; Akre et al. 1980). The wasps have in the interim remained there until they resurfaced in Maryland in 1968 (Morse et al. 1977) and have since spread to the south as well as to the west coast. The first recording of *V. germanica* in Canada was in Ontario in 1971 (Galloway and Preston 1982; Buck et al. 2008). It is suspected that the first propagules (inseminated queens) introduced into New Zealand in 1945 became established populations, originating from the United Kingdom (Thomas 1960). From there they quickly spread through the north and south island of New Zealand. In 1959 the species was discovered in Tasmania (Spradbery and Maywald 1992), and in 1977 and 1978 the first nests were recorded in the Australian ports of New South Wales, Victoria, South Australia and Western Australia (Spradbery and Dvořák 2010). The species currently occur in the southeastern half of New South Wales, and near Adelaide and Perth (Spradbery and Maywald 1992). It is believed that *V. germanica* was introduced into Argentina via Chile. The wasp became established in Santiago in 1974 (Edwards 1984) and was first recorded in Argentina in 1978 at Neuquen (D'Adamo et al. 2002). The invasion path and life history traits of *V. germanica* in its endemic and invaded ranges of New Zealand and Australia have been intensively studied, but little research has been conducted on the population dynamics of this wasp locally since its introduction into South Africa (Allsopp, pers. comm. 2014).

It is presumed that the first *V. germanica* wasps that was introduced into South Africa and recorded in 1974, hail from either their native range in the Northern Hemisphere or from countries in the Southern Hemisphere that were invaded. There is evidence for the introduction of *V. germanica* from the United Kingdom via air cargo into New Zealand during 1944/45. Remains of dead queens that presumably hibernated in the wooden crates, which contained aircraft spare parts, were discovered near an Air Force depot in Hamilton, New Zealand (Thomas 1960). *Vespula germanica* could have been introduced from Europe into South Africa in a similar way. On the other hand, the South African population could also have a Southern Hemisphere origin. Reports suggest that Tasmania was invaded

in 1959, followed by South Africa in 1974 and that queens established in Australian port capitals during 1977/78, after consignments of timber were shipped from New Zealand to Sydney, and the first interception of *V. germanica* occurred in 1954 (Chadwick and Nikitin 1969). Therefore, *V. germanica* could also have been transported to South Africa by sea. If that was the case, it would be more likely that fertilised queens introduced via a shipping route into South Africa would have originated from the Southern Hemisphere, rather than the Northern Hemisphere. By sea, hibernating fertilised queens arriving from the Northern Hemisphere to the Southern Hemisphere would come out of diapause as it warmed up before the equator and probably starve or suffer severe stress en route and die. Nevertheless, fertilised queens could be transported from the East to the West in the Southern Hemisphere via shipping routes during the winter months. It is imperative to identify routes of invasion and spread dynamics of an invasive species if the aim is to prevent further entry and to employ management strategies, which is one of the objectives of this project (Hulme 2009). To address this void in local knowledge, geometric morphometric analyses were employed to infer possible origin and invasion routes (Gargan et al. 2016) of *V. germanica* in South Africa, by assessing variation in wing shape.

Whereas molecular techniques can be expensive, geometric morphometrics is a simple, less expensive and very accurate tool to study the size and shape of organisms using powerful statistical analyses which can answer similar questions to those answered by molecular techniques (Adams et al. 2004; Dujardin 2008; De Morais et al. 2010; Galatius et al. 2012). Many studies have characterised populations within species according to variation of shape to establish geographic origin and population structure (Haas and Tolley 1998; Nielsen et al. 1999; Roggero and d'Entreves 2005; Bischoff et al. 2009, Alghamdi et al. 2013; Nunes et al. 2013; Lester et al. 2014; Prado-Silva et al. 2016).

In this study, the shape of *V. germanica* individuals was represented by landmarks on wings (points on vein intersections), which are homologous (Webster and Sheets 2010). Landmark-based geometric morphometrics as a tool to study wing shape variation among populations, has shown to be informative for use in studies with other insects (Zahiri et al. 2006; Dujardin 2008, 2011; Vidal and Suesdek 2012; Lashkari et al. 2013; Demari-Silva et al. 2014; Hidalgo et al. 2015) as well as social wasps (Abbasi et al. 2009; Lester et al. 2014; Perrard et al. 2015).

The purpose of this study was the following: firstly, to compare the intraspecific shape differences between samples from Kirstenbosch in South Africa to samples from the suspected regions of origin, namely Europe (France), Australia and New Zealand. The hypothesis is that the South African *V. germanica* population would be morphologically more similar to populations from either France or Australia and New Zealand compared to populations from Argentina. Secondly, to explore morphological heterogeneity among samples which were collected from nine different geographic locations within South Africa. The results could give an indication of the invasion pathway followed locally (Bischoff et al. 2009; Nunes et al. 2012; Prado-Silva et al. 2016). Lastly, to determine whether there is a direct correlation between the difference in shape of wasp samples and the geographic distance among the locations that they were collected from, which could contribute to understanding the population structure of *V. germanica* in South Africa (Goodisman et al. 2001; Garnier et al. 2004; D'Anatro and Lessa 2006; Lashkari et al. 2013).

Materials and Methods

Sample and collection sites

Vespula germanica workers (females) were collected over a period of two years (2013-2015), from thirteen distinct locations around the world (Fig. 1 and Table 1). At each location 65 wasps were caught and a total of 845 individual wasps were used in the analyses. All specimens from a specific location were collected from the same nest. The sampling area in the Western Cape Province covered a large part of the known distribution of *V. germanica* in South Africa (Chapter 1). Wasps were collected using sweeping nets and preserved in 99 % EtOH until slide-mounted. Specimens were sexed by counting the number of gastral and antennal segments under a stereomicroscope. Researchers from New Zealand, Australia, France and Argentina were contacted and asked to post wasp samples, preserved in EtOH (99.9 % purity), collected from a single nest, in sealed containers.
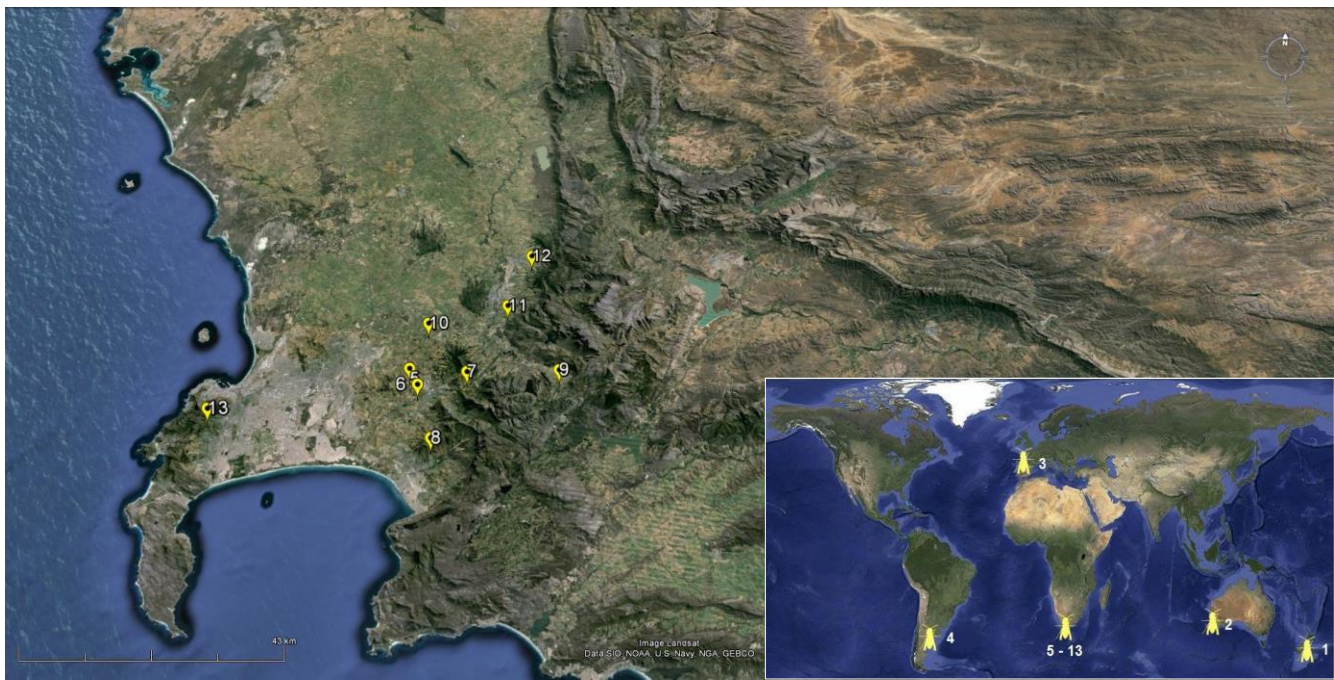
Fig. 1 Map indicating the location of the nine sampling sites within the Western Cape, South Africa as well as the location of specimens received from overseas.

Table 1 Geographical locations and coordinates of female *Vespula germanica* samples collected.

Number	Location	Coordinates
1	New Zealand, Christchurch	43°17'28.89"S, 172°11'48.03"E
2	Australia, Perth	31°59'9.39"S, 115°57'41.10"E
3	France, Paris	48°50'27.8"N, 2°21'34.2"E
4	Argentina, Rio Negro	43°17'28.89"S, 172°11'48.03"E
5	Stellenbosch 1	33°54'59.73"S, 18°48'59.45"E
6	Stellenbosch 2	33°56'58.91"S, 18°50'15.14"E
7	Banhoek	33°55'31.91"S, 18°56'1.24"E
8	Somerset West	34°03'19.01"S, 18°52'45.99"E
9	Franschhoek	33°55'21.35"S, 19°07'07.10"E
10	Elsenburg	33°49'12.38"S, 18°50'26.5"E
11	Paarl	33°46'53.92"S, 19°0'4.24"E
12	Wellington	33°40'3.54"S, 19°2'31.68"E
13	Kirstenbosch, Cape Town	33°59'29.78"S, 18°25'43.64"E

Preparation of specimens

Both left (n = 65) and right (n = 65) forewings of newly collected individuals from a specific location were detached from the thorax at its base and mounted on a microscope slide (3 x 1 inch)(75 x 25 mm) in 2 drops of mounting medium (Entellan) and covered with a cover slip (9 x 35 mm). Each mounted wing was photographed with a Zeiss Stemi 2000-C stereo-microscope and a scale bar was placed on each image. Images were saved in JPG format and labeled according to location, individual number, and wing side (ex. Arg0001L). To prepare images for digitization of landmarks, tpsUtil software Version 1.58 (Rohlf 2008) was used to build tps files of the images.

Landmark data collection

Seventeen Type 1 (Bookstein 1991; Zelditch et al. 2004) landmarks, located at vein intersections (Fig. 2) were chosen due to their reliability as homologous structures and their capacity to describe wing shape (Abbasi et al. 2009; Perrard et al. 2015) and digitized, in the same order, onto every wing using the programme tpsDig Version 1.40 (Rohlf 2006). A total of 1690 images of left and right wasp wings were digitized. Imaging and digitization were conducted by the same person. A consensus file was created using tpsRELW Version 1.53, which aligned raw coordinate data prior to further analyses (Rohlf 2005). To convert the consensus file containing coordinate data, tpsUTIL was used and the file saved as a CSV file which could be processed in further analyses as detailed below.
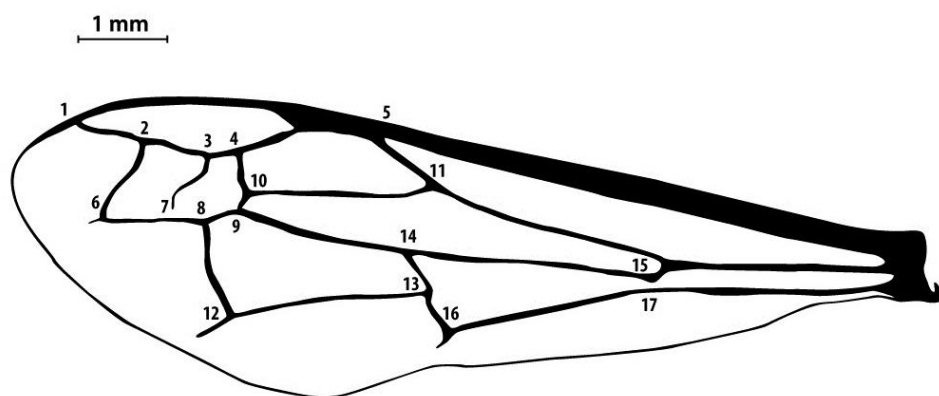
Fig. 2 A representation of the seventeen landmarks defined by vein intersections on the surface of a *Vespula germanica* forewing.

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Data analyses

To ensure that changes in wing shape and size was not significantly influenced by imaging and digitization errors, both left and right wings of an individual were chosen at random in a subset of 26 specimens. Wings were photographed twice (to quantify imaging error) and each image copied and again digitized (to quantify digitizing error). Each of the images was digitized by the same observer. A Procrustes ANOVA (Klingenberg and McIntyre 1998; Klingenberg et al. 2002) was, therefore, performed to quantify measurement error on two levels, imaging error and digitizing error, in relation to random asymmetry (individual-by-side interaction). A separate dataset was therefore used for the analyses of measurement error.

The programme MorphoJ Version 1.05f (Klingenberg 2011) was used for further analyses of the total dataset. To extract shape information from landmark configurations, components of variation such as size, position and orientation were removed by performing a full Procrustes fit (Dryden and Mardia 1998) and data were aligned by principal axes into a common coordinate system. To determine the presence of allometry and its influence on shape variation, a multivariate regression analysis was conducted of shape on centroid size (Loy et al. 1996; Drake and Klingenberg 2008; Sidlauskas et al. 2011), with centroid size as independent variable and Procrustes coordinates as dependent variable. The significance of allometric influence was assessed by a permutation test with 10 000 randomizations. To correct for effects of allometry, the ouput dataset containing the residuals from the regression of shape on centroid size, was used for further analyses.

Since a set of landmarks on both wings were digitized on each wasp, the landmark coordinates were averaged between wings so that a single vector of average wing shape for every individual wasp could be obtained. To analyze the variation among all individuals in the dataset, principal component analysis (PCA) was used to create a new set of variables (PCs), which is computed from a covariance matrix (Rohlf 1993, 1996). Eigenvalues are extracted and the relative amount of variance each eigenvalue accounts for, is expressed as a percentage of total shape variance. To compare overall shape feature differences of wasp wings within and between different countries, canonical variate analysis (CVA) was used as ordination method and results were reported as Mahalanobis distances. To assess the significance between these distances, permutation tests (10 000 permutation randomizations) were performed. To determine whether a correlation existed between the geographic distance from various locations within South Africa and the wing shape differences of *V. germanica* samples, the Mahalanobis distance (dependent variable derived from shape variation) was plotted against geographic distance (independent variable) and a regression analysis was conducted.

Results

Procrustes ANOVA for measurement error

The mean squares (MS) for imaging error and digitization error were smaller than the individual x side interaction, which indicate that the shape variation present in the data as a result of imaging and digitization does not contribute much to variation in size and shape and is therefore negligible (Table 2).

Table 2 Procrustes ANOVA analysis for the assessment of measurement error at two nested levels for forewing shape and size of *Vespula germanica*.

	SS	MS	df	F	P
Centroid size					
Effect					
Individual	0.000391	0.000016	25	17.83	< 0.0001
Side	0.000000	0.000000	1	0.00	0.9926
Individual * Side	0.000022	**0.000001**	25	3.07	0.0003
Imaging error	0.000015	**0.000000**	52	27.19	< 0.0001
Digitizing error	0.000001	**0.000000**	104		
Shape					
Effect					
Individual	0.03485996	0.0000464799	750	10.12	< 0.0001
Side	0.00075385	0.0000251282	30	5.47	< 0.0001
Individual * Side	0.00344369	**0.0000045916**	750	8.82	< 0.0001
Imaging error	0.00081212	**0.0000005206**	1560	3.66	< 0.0001
Digitizing error	0.00044383	**0.0000001423**	3120		

Allometry

The multivariate regression analysis showed a strong relationship between the regression scores and centroid size. A 12.9 % proportion of shape variance was explained by size, which was statistically significant (p<0.0001). Therefore, data was corrected for allometric variation by removal of the allometric effect (Klingenberg 1996; Dujardin et al. 2001).

Principle component analysis (PCA)

The first three PCs accounted for 48.37 % of overall variation in wing shape contained in the dataset. The total variance, that indicates the amount of shape variation in the sample, was 0.00016865. PC1 accounted for 18.64 % of the variation, PC2 for 17.24 % and PC3 for 12.49 %. Groups could be distinguished according to collection site in the PCA ordination plot (Fig. 3). Wasp samples from Argentina were placed in the top, right-hand quadrant and samples from New Zealand and Australia in the bottom right-hand quadrant. Samples from France and South Africa were placed mainly in the left-hand quadrant.
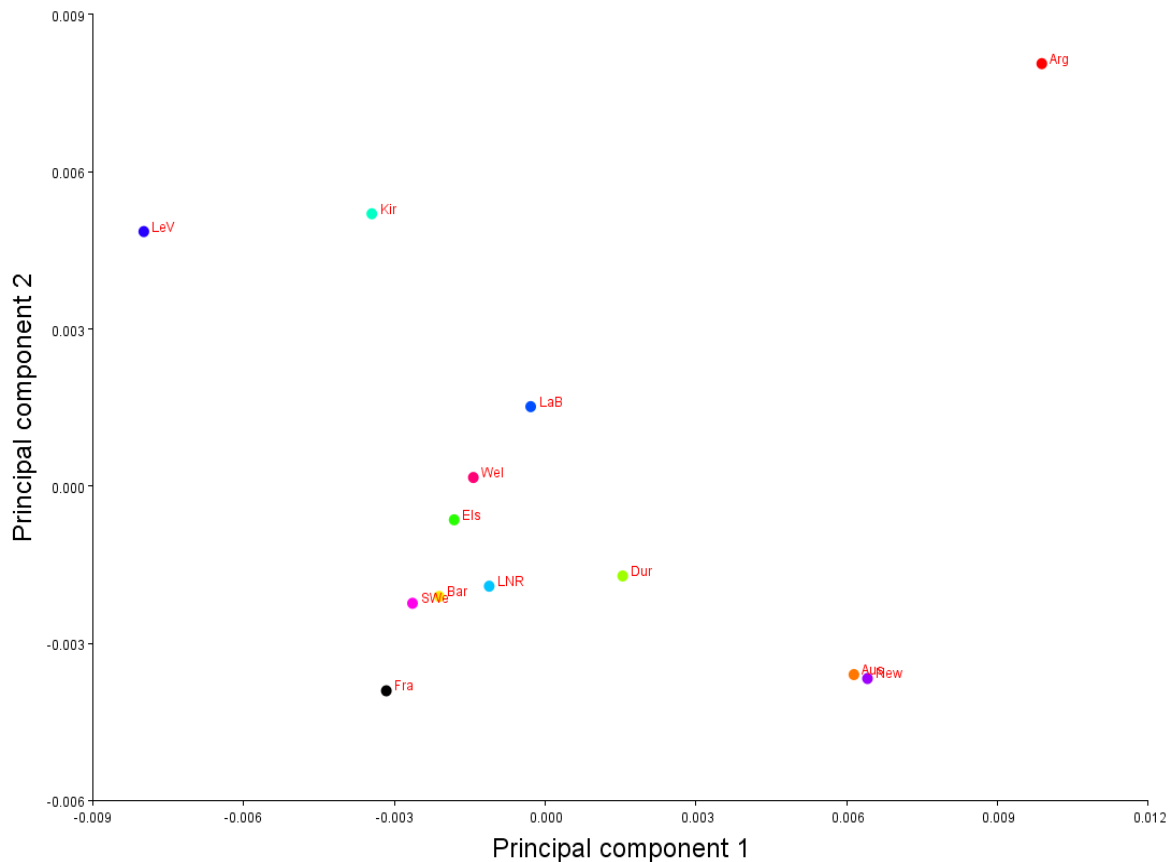
Fig. 3 A PCA scatterplot, showing the arrangement of *Vespula germanica* populations based on frontwing shape, averaged by location, in the multivariate tangent shape space. LeV - Stellenbosch 1, SWe – Somerset West, LNR – Stellenbosch 2, Bar – Banhoek, LaB – Franschhoek, Wel – Wellington, Fra – France, Els – Elsenburg, Dur – Paarl, Kir – Kirstenbosch, Aus – Australia, New – New Zealand, Arg – Argentina.

The shape changes associated with the first PC in the negative direction (Fig. 4B), showed a slight expansion of the wing horizontally, especially between landmark LM5 and LM4. In the positive direction (Fig. 4A) a slight horizontal compression of the wing occurred. The cross veins between LM2 and LM6, LM3 and LM 7, LM4 and LM10, LM8 and LM 12, and LM14, 13 and 16, mostly changed the width of cells between these cross veins. In the negative direction, the three widths of cells decreased, whereas in the positive direction the cells expanded.

A
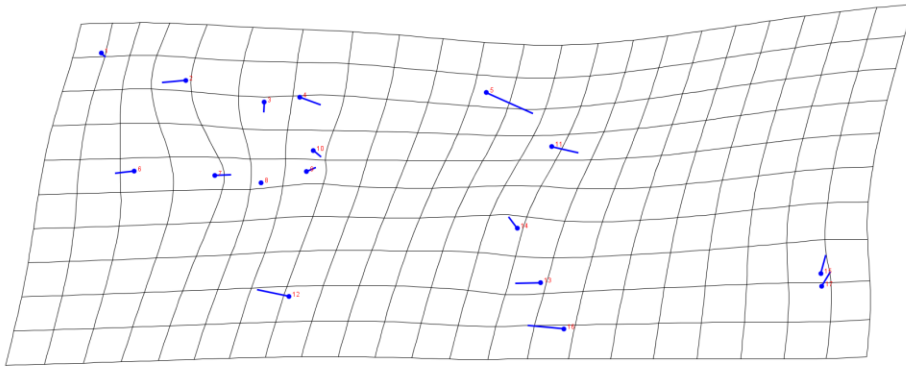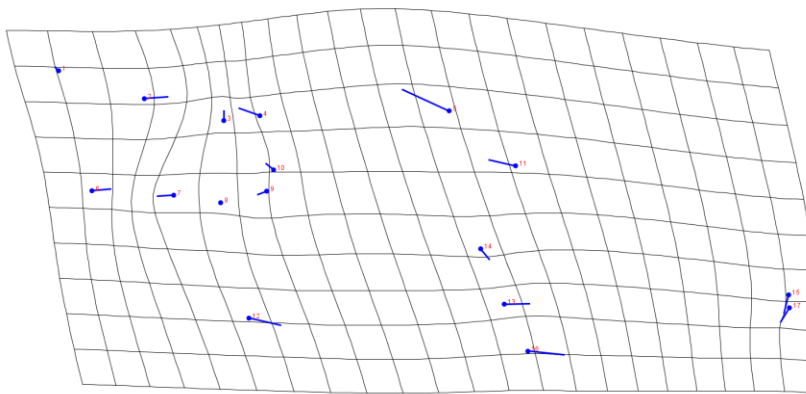
B

Fig. 4 Visualizations produced using the thin-plate spline of wing shape change for the first PC on a transformation grid corresponding to a Procrustes distance of 0.1 units in a positive (A) and negative (B) direction. Location of 17 landmarks (solid dots) indicates average landmark positions and lines represent the landmark shifts associated with the respective PC change in various regions of the wing.

Canonical variate analysis (CVA)

The first three CVs accounted for 57.922 % of the amount of relative between-group variation. CV1 accounted for 28.284 %, CV2 for 18.954 % and CV3 for 10.684 %. In Fig. 5, the 90 % confidence ellipses for each geographic location are plotted for ease of distinguishing groups. Clear clustering of samples from similar localities can be observed in the figure. Along the CV2 axes, samples from Argentina were separated from those of all other localities, whereas New Zealand and Kirstenbosch

samples were most differentiated based on the shape changes associated with CV1. Samples collected from South African localities grouped together and overlapping occurred. Samples from France overlapped with all South African samples, except for samples from Kirstenbosch. However, based on the Mahalabanobis square distances (Table 5), samples from Kirstenbosch were still most similar in shape to samples from France, compared to the other localities overseas. Shape differences between samples from New Zealand and Australia were lower compared to that between Australia and France. Even though some groups clustered together, results from the permutation test showed that there were still significant differences among all groups ($p<0.0001$).
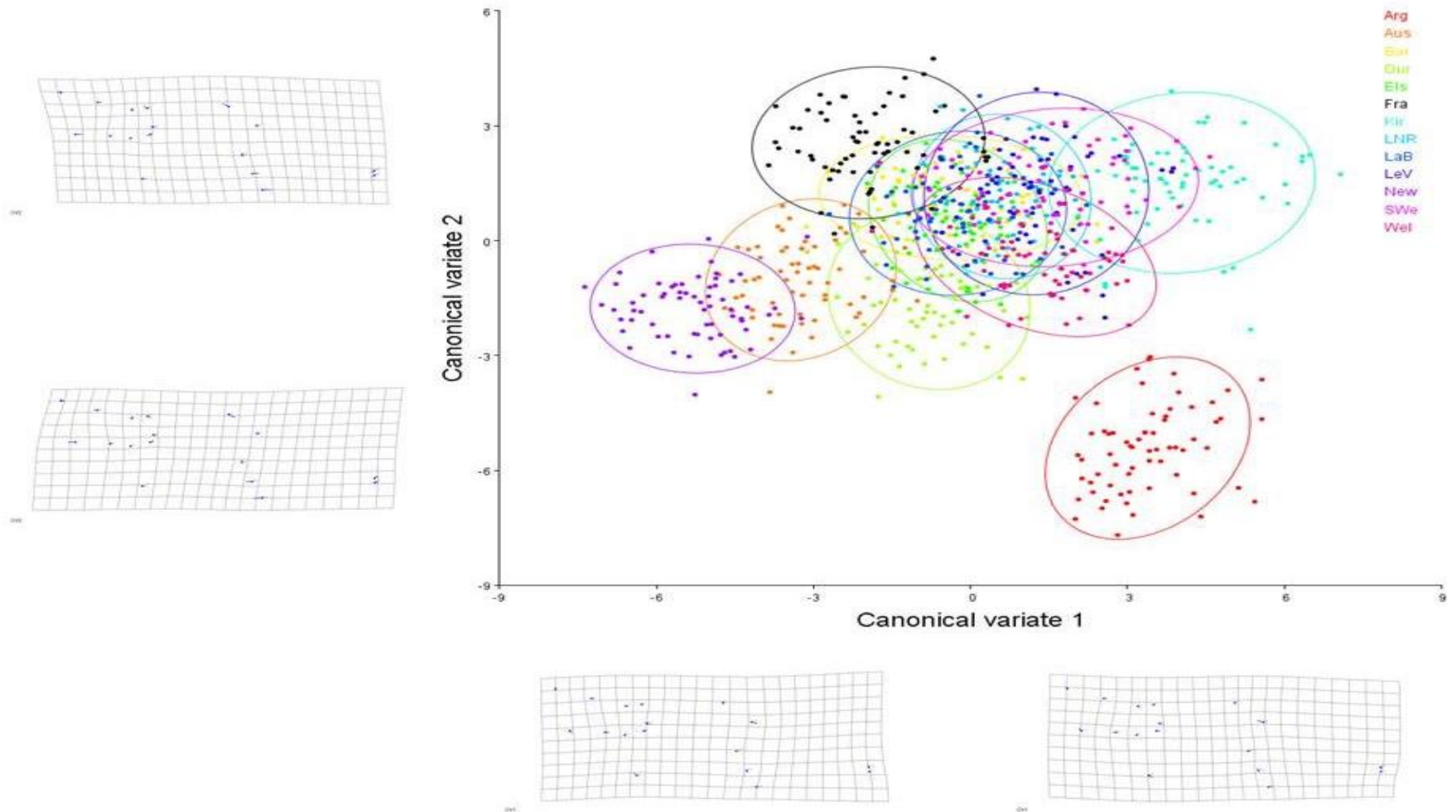
Fig. 5 The CVA scatter plot for the first two canonical variants showing the relative position of *Vespula germanica* individuals in terms of shape variation, collected from the thirteen different locations, represented by coloured dots. Shape changes in a positive and negative direction of forewings along the CV1 and CV2 axis are represented as transformation grids amplified to a Mahalanobis distance of 20 units for better visualization. Arg – Argentina, Aus – Australia , Bar – Banhoek, Dur – Paarl, Els – Elsenburg, Fra – France, Kir – Kirstenbosch, LNR – Stellenbosch 2, LaB – Franschhoek, LeV - Stellenbosch 1, New – New Zealand, SWe – Somerset West, Wel – Wellington.

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Mahalanobis square distances among groups (Table 5) ranged from 4.4292 to 10.4149, where the smallest morphometric distance was between samples from Elsenberg and Franschhoek (4.4292). The greatest morphometric distance was between Kirstenbosch and New Zealand (10.4149). It was noted that the furthest Mahalanobis distance was also the furthest (17 098 km) geographic distance between the respective groups. Permutation tests (10 000 permutation randomizations) revealed a significant difference between each of the locations, showing that samples from the various sampling sites were highly distinct (p<0.0001).

Table 5 Morphological distances of forewings between populations of *Vespula germanica* from different geographic locations, using the Mahalanobis distances matrix. The numbers in bold are the shortest and longest distances between two collection sites, respectively.

Locations	1	2	3	4	5	6	7	8	9	10	11	12
2	9.0096											
3	8.5521	6.8337										
4	7.7356	5.9048	6.1149									
5	8.1459	5.8908	4.8186	5.1790								
6	9.9762	6.5503	6.3930	6.8677	6.1782							
7	8.0594	8.3566	6.9068	7.6011	6.8817	8.0421						
8	7.8306	6.3378	4.4413	6.4963	5.0174	5.9148	5.7217					
9	8.0528	5.4022	5.1905	5.4535	**4.4292**	5.5461	5.5419	5.2598				
10	8.5721	7.8261	5.8520	6.6754	5.7354	7.1626	6.2669	6.0083	5.4331			
11	9.8994	5.4850	7.0700	7.0442	7.5020	7.2749	**10.4149**	7.6982	6.8079	8.5182		
12	7.8187	7.2377	5.6648	6.5214	5.5255	5.5362	5.4445	4.7340	5.3181	6.8430	8.6348	
13	7.2757	6.8751	6.0395	4.8148	5.1319	6.5629	6.0140	6.2040	4.8064	5.9313	8.1518	5.1955

1 – Argentina, 2 - Australia, 3 – Banhoek, 4 – Paarl, 5 – Elsenburg, 6 – France, 7 – Kirstenbosch, 8 – Stellenbosch 2, 9 – Franschhoek, 10 – Stellenbosch 1, 11 – New Zealand, 12 – Somerset West, 13 – Wellington.

Isolation by distance

Overall, a significant correlation (Fig. 6, Table 6) was found between the geographic distance and Mahalanobis distance ($p<0.0001$) between wasps, which indicates that as the geographic distance between sampling sites increases, the wing shape differentiation increases as well. However, when analysing the correlation between these two factors separately for each location, not all correlations were significant (Table 6) and the slope of regression varied among locations. In all locations, except one, a positively correlated relationship was observed. Samples from Kirstenbosch showed a slight negative correlation between wing shape and geographic distance.
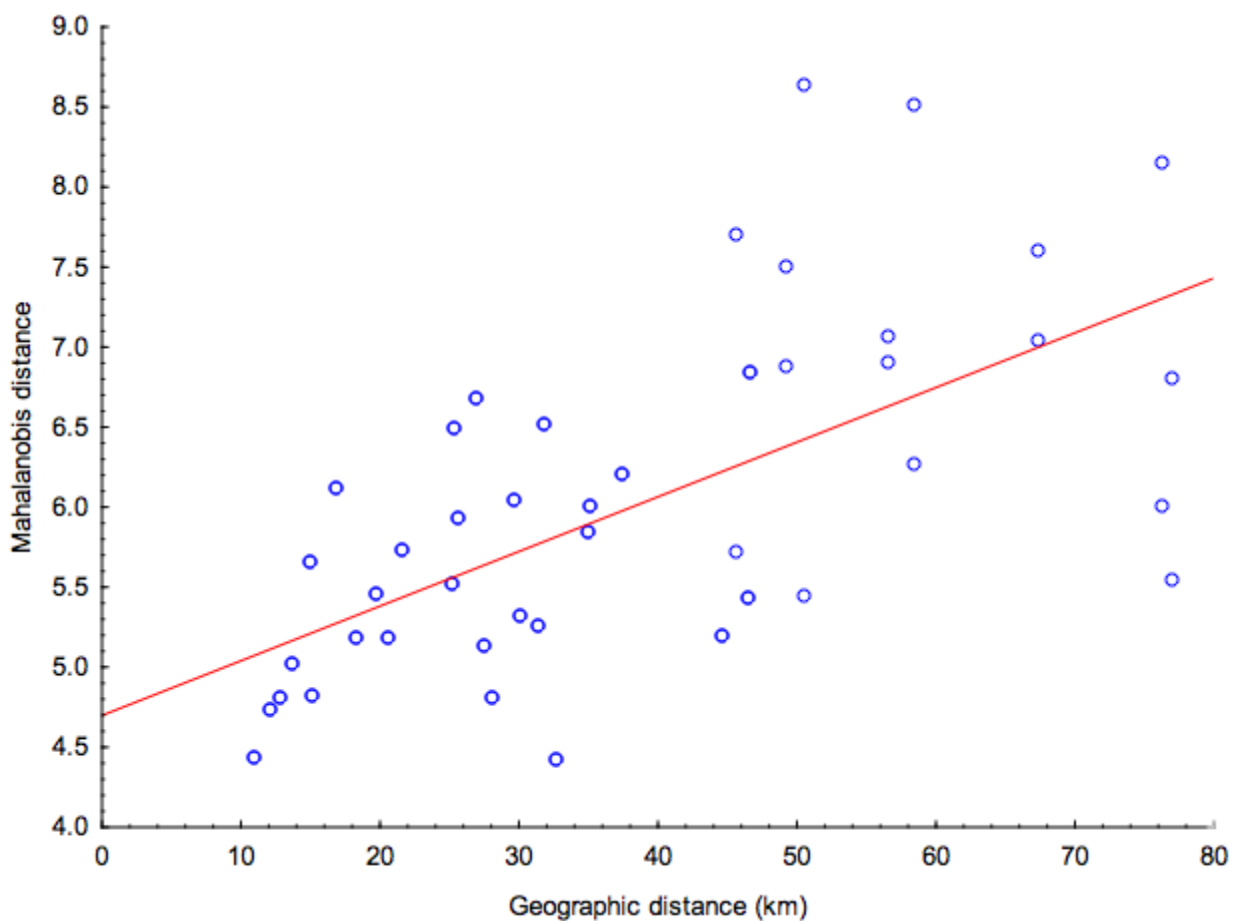
Fig. 6 The correlation between the Mahalanobis distance of *Vespula germanica* forewings and the geographic distance (km) of samples collected from all the locations within the distribution range of this wasp in the Western Cape Province.

Table 6 The correlation coefficient (r) and p-value for each location in the regression analysis conducted on the geographic distance between wasp samples and the Mahalanobis distance.

Location	r	p
Stellenbosch 1	0.162	0.702
Stellenbosch 2	0.707	0.049
Banhoek	0.801	0.017
Somerset West	0.388	0.341
Franschhoek	0.348	0.399
Elsenburg	0.640	0.087
Paarl	0.840	0.009
Wellington	0.459	0.252
Kirstenbosch	-0.311	0.454

Invasion pathway

Based on the Mahalanobis distance, the results suggest that from Kirstenbosch, which is assumed to be the original port of entry, the wasps spread to Somerset-West, visually represented by Figure 7 in Appendix A. Samples from Kirstenbosch (# 13) were most similar in shape out of all the South African populations to that of the Somerset West (# 8) samples. Samples from Somerset West were most similar to samples from Stellenbosch 2 (# 6) and Stellenbosch 2 samples most similar to Banhoek (# 7) samples. In turn, Banhoek samples were also most similar to Stellenbosch 2 samples, followed in similarity by samples from Elsenburg (#10). Elsenburg samples were most similar to Franschhoek (# 9) samples. After that, by solely relying on morphology, the geographic traceability of spread becomes less clear. As indicated on the map in Appendix A, the wasps moved along the Hottentots Holland mountain range toward Franschhoek and not across it.

Discussion

The suspected origin of *V. germanica* wasps which were accidently introduced into South Africa 42 years ago, have not yet been confirmed through quantitative studies (Whitehead 1975; Whitehead and Prins 1975; Tribe and Richardson 1994; Giliomee 2011). Based on reports that the first specimen of *V.*

germanica found in South Africa was from Kirstenbosch (Whitehead 1975; Whitehead and Prins 1975), it is assumed that the invasion spread from this point. In 1974, a specimen identified as *V. germanica* was also found at a container depot near Irene (25°52"S, 28°13"E), which is in the northern part of the country in Gauteng Province and could have been as a result of a second invasion (Tribe and Richardson 1994). However, since then, there have been no new documented, confirmed reports of *V. germanica* from northern parts of the country. In this study, the first aim was to derive the area of origin of invasive *V. germanica* populations in South Africa.

The PCA and CVA analyses that were conducted on *V. germanica* samples visually presented clear variability and separation of groups according to geographic origin, even at a geographic distance as small as 10 km (between Banhoek and Stellenbosch 2) and after removal of the allometric component. The association of groups that were expected to be genetically closer, according to known Australasian invasion pathways and historical invasion records (Thomas 1960; Smithers and Holloway 1977; Spradbery and Dvořák 2010), could be an indication that wing morphology is a reliable characteristic in determining the origin of wasp propagules in this study. The Australian population grouped closely to the New Zealand population, whereas the population from Argentina was sharply separated on both the CV1 and CV2 axes from all the other populations.

Samples from Kirstenbosch differed significantly (p<0.0001) from all of the overseas localities, but were closest to the group from France, as opposed to larger shape differences present when compared to the populations from New Zealand, Australia and Argentina. This reveals that wasps from Kirstenbosch most likely stemmed from Europe rather than Australia, New Zealand or Argentina. Wasps from Kirstenbosch differed mostly from the French samples in terms of the shape changes associated with CV1, which accounted for most of the variation.

Vespula germanica can naturally disperse between 30-70 km per year in favourable habitats, due to their strong flying capabilities (Edwards 1980, Crosland 1991; Ross and Carpenter 1991; Moller et al. 1991; Moller 1996), and through human-aided transport. Hibernating, inseminated queens can be moved vast distances, which have most likely resulted in the jump dispersal via aircraft (Masciocchi and Corley 2012) of wasps from Europe to South Africa.

Obtaining wasp samples from areas outside of South Africa underrepresented in this study, such as North America, Canada, North Africa and Asia, where both native and invaded ranges of *V. germanica* can be found, would be particularly instructive in confirming the origin and determining the relation of South African *V. germanica* populations to populations in the rest of the world.

The second aim was to elucidate the possible invasion pathway that was followed after introduction into South Africa by comparing the wing shape of samples from different locations within the Western Cape Province. The distances between sampling sites ranged from 10.9 km to 77 km and encompassed most of the currently known distribution of *V. germanica* in South Africa. Samples from various sites within this geographic perimeter, grouped together. This result is supported by the uncharacteristically slow dispersal of *V. germanica* in South Africa (Veldtman et al. 2012; Allsopp, pers. comm. 2014; Haupt 2014), which will limit the degree of genetic exchange that takes place and accordingly influences the morphological variation among local wasp populations (Garnier et al. 2004). It could also allude to the possibility that distribution of *V. germanica* in the Western Cape occurred by means of natural dispersal only.

Even though samples from South Africa grouped together on the CVA plot, shape differentiation among all of the local localities, as measured by the Mahalanobis distances, was significant. This variation could potentially be attributed to a founder effect, such as a population bottleneck that occurred when *V. germanica* propagules were introduced into South Africa (Goodisman et al. 2001; Allsopp, pers. comm. 2014). Since the wasps were accidently introduced (Whitehead and Prins 1975; Tribe and Richardson 1994), it is probable that a small number of propagules founded the first local population. A lowered genetic diversity within a population gene pool is expected when only a few individuals are introduced into a new area (Chakraborty and Nei 1977; Luikart et al. 1998; Goodisman et al. 2001; Sakai et al. 2001; Dlugosch and Parker 2008). This is an especially typical outcome with invasive social insects (Chapman and Bourke 2001; Ellis et al. 2006; Gruber et al. 2012; Lester et al. 2014). In this case, the founder effect could have been exacerbated by the annual, fluctuating population densities of *V. germanica* and a tendency of newly introduced populations to remain small for a few years, before undergoing exponential growth (Archer 1980; Crosland 1991; Harris and Beggs 1995). In addition, the CFR, where *V. germanica* populations currently occur, has shown to be a marginal habitat for these wasps (Tribe and Richardson 1994), which would impose additional stress upon newly introduced populations. The combination of all these factors could have caused population bottlenecks not only during the initial introduction, but also during subsequent spread (Goodisman et al. 2001). Shape differentiation among local populations could also have been influenced by the sampling protocol followed. Since samples at each locality were collected from a single nest, the shape variation obtained could be due to large natural variation in wing shape of wasps collected from different nests within the same locality. To confirm this, future studies should collect wasps from

various nests representing a single locality, determine the shape variation among localities and compare it to the shape variation obtained if wasps were collected from a single nest that represented a specific locality. If there is a significant difference between the variations, the variation in wasp wing shape among local nests within a single locality should be taken into consideration when interpreting the Mahalanobis distances obtained within and between local and international samples.

Phenotypic characteristics are influenced by both environmental and genetic factors (Dujardin and Le Pont 2004). However, in an effort to distinguish between wasp and hornet species, Perrard et al. (2014) concluded that genetics had a stronger influence on the wing shape of hornets and wasps than other biotic and abiotic factors. In addition, geometric morphometrics have successfully been used to confirm genetic variability among populations of other insects (Mendes et al. 2007; Francoy et al. 2011; Gargan et al. 2016). Therefore, the variation in wings that was found among local populations in this study, could, arguably, be mainly attributed to changes at the genetic level and be thus explained. Due to the relatively confined spread of *V. germanica*, sampling sites were not further than 77 km from each other. Extreme differences in climatic parameters were not the norm. Its influence on the morphological characteristics of wasps might therefore not have been as prominent as the influence of genetic factors.

In general, as population numbers of an invasive species that experienced an initial bottleneck upon entry into a new environment gradually increase, random mutations occur. Combined with low genetic diversity, this could lead to strong genetic drift, consequently increasing genetic differentiation among populations (Chakraborty and Nei 1977; Sakai et al. 2001). Assuming that a small number of wasps, containing the genetic signature of the original invasive population, were introduced into South Africa, not all genotypes from the original invasive population would be represented in the new populations which had spread to different regions. These populations will therefore differ in genetic makeup. If sufficient time has passed, it will allow for morphometric variability to develop which could potentially explain the differences in wing shape among local *V. germanica* populations.

Similar findings were obtained in a study by Chau et al. (2015), where strong patterns of genetic differentiation were observed in populations of the social wasp *Vespula pensylvanica,* in the introduced range, as opposed to little genetic structure within its native range. Goodisman et al. (2001) found that the genetic structure of invasive *V. germanica* populations from four different localities in Australia

varied substantially among populations, which supported their hypothesis that population bottlenecks occurred during establishment.

The combined effects of behavioural traits of invasive *V. germanica* populations, characteristics of the invaded environment in South Africa, and the suspected genetic bottlenecks that occurred, indirectly depicted by morphological differentiation among localities, would all have contributed to reduce the dispersal rate of these wasps, which, to date, has been evidently slow in South Africa (Tribe and Richardson 1994; Haupt 2014).

In this study, the wing shape of wasps from Kirstenbosch differed more from any of the other samples collected from within South Africa. The difference was more pronounced than among the other samples. This is consistent with the theory that wasps were introduced into South Africa via Kirstenbosch. *Vespula germanica* is known to be associated with humans (Spradbery and Dvořák 2010), and human habitation was present along the invasion route followed, and not in the mountainous area, which is a nature reserve. This pathway was potentially the easiest route for dispersal. Future predictions of *V. germanica* dispersal in South Africa and management practices to curb further spread, can now be based on this newly generated knowledge of the potential route of invasion that was followed. Mountain ranges were suspected to serve as a natural barrier for *V. germanica* movement and it was confirmed by this study. Accordingly, this could assist in estimating routes that will be followed if steady range expansion continues. Based on these estimations, control efforts should be implemented at the periphery of the distribution range. Since it is suggested that the wasps are spreading mainly by natural means, implementing control practices on the fringes might be the most well suited approach. In addition, the presence of this wasp, especially within urban areas where they are in close association with humans and mostly occur, will influence what type of control agents are selected. Focusing on control agents that are not harmful to humans will have to be a priority.

The third aim of this study was to determine the correlation between geographic distance and shape variation of samples collected from various localities within South Africa. There was an overall significant positive correlation between the shape differences and geographic distances, which means that as the geographic distance increases between sampling sites, the morphological similarity of wasps from the respective sites, decreases. This is known as isolation-by-distance (Hartl and Clark 1989). This suggests that the distance between wasp populations could be a good indication of the level of shape differentiation that will occur (Kingsolver et al. 2007; Lashkari et al. 2013). This supports the hypothesis mentioned earlier that *V. germanica* mainly spread by means of natural dispersal, which

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agrees with the pattern of isolation-by-distance. It could point to very little interaction occurring among local populations, even at small geographic distances. This is consistent with a study conducted by Goodisman et al. (2001) that found a positive correlation between distance and restricted gene flow in *V. germanica.* The one case (Kirstenbosch) where a negative correlation occurred between these two factors, could allude to the possibility that multiple introductions occurred via Kirstenbosch and might still be occurring. However, further research is necessary to confirm this hypothesis.

This study identified the potential origin of *V. germanica* wasps in South Africa and the invasion pathway followed. To confirm these results, the concomitant use of genetic analysis with the available morphological data is suggested (Vidal and Suesdek 2012; Lashcarie et al. 2013; Gargan et al. 2016).

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Appendix A

Fig. 7 Invasion pathway of *Vespula germanica* in the Western Cape of South Africa as determined by geometric morphometric analysis. Yellow arrows indicate the continuous route followed from the area of introduction, Kirstenbosch (13), to Franschhoek (9).

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Chapter 5

Potential attractants for *Vespula germanica* and *Polistes dominula* workers collected from the Western Cape Province in South Africa

Abstract

The attractiveness of several carbohydrate and protein-based baits/lures were compared in a Y-tube olfactory bioassay to assess the response to it of two locally invasive wasp species, *Vespula germanica* (Fabricius) and *Polistes dominula* (Christ) foragers, collected in the field. The aim was to find effective baits/lures that could be used to monitor and control populations of these two wasp species in the Western Cape Province. *Vespula germanica* responded most favourably to cooked ham and least favourably to Minute Maid[®], whereas *P. dominula* foragers preferred odour cues derived from their own nest above all the other baits/lures that were tested. Additionally, it was found that the attractiveness of baits/lures that were tested, and the response time of wasps to its presence in the Y-tube olfactometer, are not directly correlated. There were also significant differences in the preference of females with developed ovaries versus undeveloped ovaries of both wasp species for baits/lures.

Introduction

There are many commercially produced products containing slow or rapid knock-down insecticides, available on the international market and said to attract social vespids to baits or traps, but no such products exist on the South African market (Landolt et al. 2000; Sackmann et al. 2001; Sackmann and Corley 2007). Invaded regions differ in terms of climate, faunal and floral diversity, wasp density, wasp feeding preferences and behavioural traits. Therefore, the efficacy of products in attracting wasps to a target under South African conditions, will not necessarily mirror the outcomes achieved in other invaded countries. The products need to be tailored for and tested in this specific environment (Spurr 1996; D'Adamo and Lozada 2005; Sackmann and Corley 2007; Gentz 2009; Haupt 2014). The

attractiveness of baits to a given wasp population is also influenced by season, immediate weather and the stage of colony development (Sackmann and Corley 2007).

Efficient baits are at the core of many monitoring and control practices. Lack of efficient baits is the main reason why control of these pestiferous wasps has remained hard to attain worldwide (Landolt et al. 2000). The wasps' olfactory system is extremely sensitive, well-developed and very specific (Krieger and Breer 1999; Field et al. 2000). This characteristic should be used to full advantage.

Obtaining baits that are highly effective under South African conditions is of even greater importance, because invasive wasp densities of *V. germanica* are relatively low (Haupt 2014), and the collection of truly representative monitoring data would entail either the deployment of a very large number of traps or the identification and development of highly attractive baits. Ultimately, the economic viability of each option would have to be investigated and compared, but practicability should also be taken into consideration. That is where improving bait efficacy, as a control and monitoring measure, might prove to be the more feasible option.

Recent field trials conducted by Haupt (2014), during which a variety of fresh baits and artificial lures had been evaluated for its attractiveness to *V. germanica*, found that fresh meat-based products, such as beef mince and smoked ham, were the most attractive baits over two seasons (2013 and 2014) in the Western Cape area. The meat-based baits consistently outperformed the artifical lures in terms of attractiveness (Haupt 2014). As for *P. dominula*, no local research regarding bait preferences have been conducted and any information concerning *P. dominula's* preference for certain olfactory cues, are scarce (Landolt et al. 2000; Landolt et al. 2014). It is known, however, that *P. dominula* is not attracted to dead protein baits (Toft and Harris 2004; Paynter and Ward 2012), whereas *V. germanica* has a scavenger nature and will readily consume dead meat, while it is also attracted to sugary substances at the beginning as well as at the end of the season (Spurr 1996; Sackmann and Corley 2007). For *V. germanica* there are a variety of substances to choose from, but the impracticality of using some baits limits many of the options.

The overarching aim of this study was to produce a list of substances, ranked, under a closed Y-tube choice olfactory setup, according to preference for it by respectively *V. germanica* and *P. dominula* foragers, that were collected in the Western Cape during sping and summer from October 2014 to March 2015. Even though artificial choice experiments in the laboratory are not as ideal as testing lures/baits in the field through trapping, it is the only way to test a relatively large number of different baits/lures in short succession. Since there are very few or no studies that have been conducted on bait/lure preferences of local *V. germanica* and *P. dominula* populations, it was imperative to test a

wide variety of lures/baits and being able to choose from a wider selection, than testing only a few and then trying to optimize potentially inferior baits/lures in the future. In addition, the Y-tube choice approach was also the only way to control the ambient environment and to obtain the required number of replicates needed to perform meaningful statistical analyses. One of the aspects that may unwittingly influence the outcome of Y-tube olfactory experiments, is that when wasps are caught in the field, they are undoubtedly very stressed and may not react in a similar manner as what they would if they were not stressed. To address this concern, wasps were caught the day before they were used in experiments and were stabilized in cages overnight.

The first objective was to determine whether various lures would illicit a positive response in local *V. germanica* and *P. dominula* foragers under controlled conditions in the laboratory. The following were tested: selected locally available attractants/lures, not specifically registered for wasp control previously; locally field-tested synthetic lures registered for use in the United States specifically for *V. germanica* control (Haupt 2014); and novel mixtures consisting of selected compounds found to be attractive to both *V. germanica* and *P. dominula* in other parts of the world according to literature. The second objective was to rank the baits/lures according to the time it took the *V. germanica* and *P. dominula* wasps to react to the olfactory cues emitted by each bait/lure. The final objective was to investigate whether females with developed ovaries and undeveloped ovaries of both wasp species react similarly towards the same bait/lure.

Material and Methods

Wasps

Vespula germanica and *P. dominula* wasps used in the olfactory experiment were all females, netted in flight during the afternoon (14:00 - 17:00) directly from nests as well as close to nests. Nests were situated in and around Stellenbosch, South Africa. Wasps were collected during the spring, summer and early autumn, from 14 October to 12 December 2014 and 23 January to 16 April 2015, and tested for their preference for various baits/lures within that period. Most foraging wasps during this time of the year are workers (females) and all wasps were dissected afterwards, to confirm the developmental status of ovaries by the presence or absence of oocytes in ovaries (Fig. 1) (Shukla et al. 2013). In the case of *V. germanica*, it is unlikely that the wasps with developed ovaries were spring queens or foundresses, since those caught and used in the olfactory trials, were much smaller in size compared to

the queens present at the time. However, the possibility do exist that the proportion of *P. dominula* females with developed ovaries that were caught, included spring queens, especially since *P. dominula* wasps were at one stage caught earlier in the season compared to *V. germanica*. But due to the difficulty in distinquishing between queen *P. dominula* wasps and *P. dominula* foragers based solely on morphological characteristics, the terms 'developed and undeveloped ovaries', were also applied to the two *P. dominula* wasp groups that were caught. Wasps were netted a day before they were used in experiments. *V. germanica* and *P. dominula* wasps were held in 15 cm^3 screened cages, separately, at an average temperature of 25° C, 50 % relative humidity (RH) and photophase of 14 L:10 D (06:00 - 20:00) provided by fluorescent lights. A honey:water solution (10:90) on cotton balls in Petri dishes was given as nutrition to which wasps had continuous access. The literature is divided in terms of whether insects are starved before olfactory choice experiments or not (Geier et al. 1999; Geier and Boeckh 1999; Hern and Dorn 2004; D'Alessandro and Turlings 2005; Lou et al. 2005; Tooker et al. 2005; D'Alessandro et al. 2006; MacKenzie et al. 2006; Robacker 2006; Proffit et al. 2007; MacKenzie et al. 2008; Piñero et al. 2008). In many of these studies, insects had continuous access to a nutrient source up until the moment they were used in trials. In this study it was also decided not to starve the wasps, because it ensured the highest survival rate of these insects under circumstances where obtaining enough individuals in the field, especially in the case of *V. germanica*, was already challenging.

Fig. 1 Developed ovaries of a *Polistes dominula* wasp, containing several oocytes.

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Baits and lures

Baits and lures were selected according to findings in previous studies in different parts of the world of what attracted *V. germanica* and *P. dominula* (Ross et al. 1984; Reid and MacDonald 1986; Landolt et al. 2000; Day and Jeanne 2001; Wegner and Jordan 2005; Dvořák and Landolt 2006; Sackmann and Corley 2007). Most baits tested were raw/fresh ingredients, such as sandwich ham, loquat, sodas and beer, to mention a few. Due to limited research that has been conducted on bait/lure preferences of invasive wasps in South Africa, there was strived to obtain a balance between the many types of baits/lures that have been documented to be attractive to social wasps. A balance was created between selecting baits/lures that worked in the Northern Hemisphere versus the Southern Hemisphere, proteinaceous versus carbohydrate baits, fermented versus unfermented baits, fresh versus synthetic baits and local material, such as loquat fruit, around which wasps were noticed to swarm in the field. *Vespula germanica* exhibit elaborate foraging behaviour and is generally attracted to both meat-based and sugar-based baits (Spurr 1996; Harris and Etheridge 2001; Gangloff-Kaufmann 2002; Sackmann and Corley 2007).

Beer have been reported to attract social wasps in Europe in the Northern Hemisphere (Dvořák 2007; Sorvari 2013) and was also one of the most effective baits used to monitor *V. germanica* populations in New Zealand in the Southern Hemisphere (Thomas 1960). Accordingly, the attraction of both *P. dominula* and *V. germanica* wasps to beer, was tested. Citrus-based sodas successfully trapped *Vespula* species in the United States (Wegner and Jordan 2005) and therefore Minute Maid® and Mountain Dew® were incorporated to represent some of the carbohydrate baits/lures that were tested. Ham was selected as protein bait, based on previous findings by Haupt (2014) conducted in the Western Cape Province of South Africa, that showed *V. germanica* wasps having a preference for it over tinned cat-food, canned tuna, pilchards, polony, salami, and various meat spreads.

Many insects are attracted to fermented baits (El-Sayed et al. 2005; Brown et al. 2014) which contains esters that are attractive to Lepidoptera (El-Sayed et al. 2005). Accordingly fermented versions of Minute Maid® and Mountain Dew®, molasses and fermented brown sugar were tested. Fermented molasses have successfully been used in New Zealand to monitor *V. germanica* populations (Thomas 1960). The volatiles associated with fermented brown sugar were shown by Brown et al. (2014) to be attractive to *V. vulgaris* in New Zealand. Acetic acid is also a product of microbial fermentation and was one of the volatiles present in the vinegar mixture that was tested (Landolt et al. 2014). Some of the other ingredients and chemical compounds in the vinegar mixture are attractants for wasps according to Landolt et al. (2000, 2014).

In an effort to add synthetic lures to the list of baits/lures tested for *V. germanica,* preliminary trials were conducted from the end of March 2015 to the beginning of April 2015 in Stellenbosch, where the attractiveness of two lures, used in the Unites States and obtained from Insect Science (Pty) Ltd, were tested and of which Haupt (2014) also field-tested one locally in 2013 and 2014. Both lures are available on the international market, but not registered for local use. The active ingredient in the one lure is 2 methyl-propanol (MP) and in the other, heptyl butyrate (HB). The lures were suspended in yellow delta traps each containing a sticky pad (Chempac (Pty) Ltd, Simondium, South Africa) and hung at ±1.5 m from the ground in a tree, which was 4 m from an active, aerial *V. germanica* nest. Each lure was tested on two separate days. An empty yellow delta trap with sticky pad, at the same height and distance from the nest, about 2 m from the baited trap, served as control in both instances. No wasps were caught in any of the traps. The lures were therefore excluded from the olfactory experiment. As a result, and due to previous research conducted by Richter and Jeanne (1985) indicating that fly lures attracted social wasps, local, commercially available synthetic lures, used in the field to control other pest insects such as codling moth *Cydia pomonella* (Lepidoptera: Tortricidae), false codling moth, *Thaumatotibia leucotreta* (Lepidoptera: Tortricidae), and fruit flies (*Ceratitis* spp.), were incorporated into this study.

Based on field observations where many *P. dominula* individuals swarmed around loquat trees and their fruit, as well as lemon tree leaves covered in woolly aphids, these materials were also included in the list of baits/lures.

To determine the effect of pheromones released by wasps that are under threat or that is present in and on their bodies as well as their nests (Landolt et al. 1995; Weston et al. 1997; D'Adamo et al. 2004; Claudia et al. 2010) mashed and agitated *V. germanica* wasps as well as *P. dominula* nests were included in the list of baits/lures.

A set of seven lures was tested for its attractiveness to *V. germanica* workers (Table 1). The number of lures that could be tested was dependant on the number of active nests that could be found in the field within the period in the season when foragers (females) are abundant.

Table 1 Response of *Vespula germanica* foragers to various baits and lures tested in a Y-tube olfactometer.

#	Bait/lure	Date performed	Bait/lure consistency	Composition and quantity
1	Minute Maid® Orange (Coca-Cola Company)	27/29 Jan 2015	Aqueous solution	200 ml
2	Vinegar mixture	31 Jan/4 Feb 2015	Aqueous solution	0.33 ml EtOH 0.33 ml Asetaldehyde 0.33ml Ethyl hexanoate 198 ml Distilled water 1 ml Apple cider vinegar
3	Sweet molasses, Nature's Choice™	5/13 Feb 2015	Aqueous solution	100 ml
4	Beer, Liefman's Frutesse® (3.8% vol)	4/5 Mrch 2015	Aqueous solution	200 ml
5	Cooked sandwich ham, Enterprise (Pty) Ltd.	10/12 Mrch 2015	Fresh, raw	50 g
6	Mashed *V. germanica* wasps	13/18 Mrch 2015	Fresh, raw	10 individuals
7	Agitated V. germanica wasps	1/16 Apr 2015	Live adult wasps	10 individuals

Polistes dominula wasps prey on invertebrates and collect nectar, but this species is not as readily trapped with baits and lures as *V. germanica* (Toft and Harris 2004; MacKenzie et al. 2006). Since no documented knowledge exists on the response of *P. dominula,* under local conditions, to any substances shown in previous studies to be attractive to these wasps in other geographic locations, a set

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of fifteen potentially attractive baits/lures were tested in a Y-tube olfactometer. To ferment Minute Maid®, Mountain Dew® and sweet molasses, 0.04 g wine yeast (Anchor VIN 7®, *Saccharomyces cerevisiae*) was added to 200 ml of each of the respective liquid lures. In the case of sweet molasses, 50 ml molasses was diluted with 250 ml distilled water before yeast was added. The yeast-inoculated liquid lures were fermented in 500 ml Erlenmeyer flasks at 15 °C for seven days, with the neck of the flask sealed with Parafilm. After the fermentation period, lures were immediatly used in olfactory trials. Brown sugar was fermented according to the method used by Brown et al. (2014), where 300 g sugar was added to 700 ml distilled water in a 500 ml Erlenmeyer flask. Anchor Bakers Yeast® (0.5 g) was added and the cylindrical neck of the flask sealed with a strip of Parafilm. The flask was placed at 20 °C for 48 h. Fermentation took place and was confirmed by the presence of bubbles against the side of the flask. The fermented content was immediately used in experimental trials after that.

Active *P. dominula* nests were more abundant in the field. Therefore, more baits and lures could be tested.

Table 2 Response of *Polistes dominula* foragers to various baits and lures tested in a Y-tube olfactometer.

| #	Bait/lure	Date performed	Bait/lure consistency	Composition and quantity
1	Minute Maid® Orange (Coca-Cola Company)	14/15 Oct 2014	Aqueous solution	200 ml
2	Mountain Dew® (PepsiCo, Inc.)	16/17 Oct 2014	Aqueous solution	200 ml
3	Fermented Minute Maid Orange (M.M) (1.5% vol)	21/22 Oct 2014	Aqueous solution	200 ml
4	Fermented Mountain Dew (M.D) (0.1% vol)	23/24 Oct 2014	Aqueous solution	200 ml
5	Loquat	28/29 Oct 2014	Fresh, raw, mashed	30 g

6	Vinegar mixture	30/31 Oct 2014	Aqueous solution	0.33 ml EtOH
				0.33 ml Asetaldehyde
				0.33 ml Ethyl hexanoate
				198 ml Distilled water
				1 ml Apple cider vinegar
7	Sweet molasses, Nature's Choice™	4/5 Oct 2014	Aqueous solution	100 ml
8	Fermented sweet molasses (FM), Nature's Choice™	13/14 Oct 2014	Aqueous solution	200 ml
9	Woolly aphids on lemon tree leaf (ILTL)	7/12 Nov 2014	Live aphids on leaf	3 leaves
10	Biolure® Fruit Fly (FF), Chempac (Pty) Ltd.	3/4 Dec 2014	Synthetic lure	211 g/kg Ammonium Acetate 91 g/kg Trimethylamine hydrochloride 3 g/kg 1,4-diaminobutane (Putrecine)
11	False codling moth (FCM) lure, Chempac (Pty) Ltd.	5/6 Dec 2014	Synthetic lure	62,5 g/kg Z-8-Dodecenyl Acetate 62,5 g/kg E-8-Dodecenyl Acetate 62,5 g/kg E-7-Dodecenyl Acetate
12	Codling moth (CM) lure, Chempac (Pty) Ltd.	10/12 Dec 2014	Synthetic lure	5,25 g/kg E8-E10 Dodecadienol
13	Fermented brown sugar (FBS)	23 Jan/18 Feb 2015	Aqueous solution	200 ml
14	*Polistes dominula* nest	10/11 Feb 2015	Fresh nest comb	6.47g nest 24 capped cells 13 larvae

| 15 | Beer, Liefman's Frutesse® | 12/13 Feb 2015 | Aqueous solution | 200 ml |

Olfactometer setup

A Y-tube olfactometer was used to assess attractiveness of the various baits and lures (Fig. 2). The setup consisted of a transparent glass tube (inner diameter 3.5 cm). The length of the stem from base to Y-junction was 19 cm and converged into two branches, where each arm was 16 cm in length. The decision mark was placed 5 cm from point of diversion of the Y-junction. PVC tubing, with a length of 30 cm and diameter of 6 mm for each tube, linked the entire system. Plastic paraffin film (Parafilm M®) was used to seal all connections between parts. The olfactory system was suspended horizontally above a white cardboard, 70 cm below three 1.2 m, red, 36 W fluorescent light tubes (Osram GmbH). The experiments were conducted in a room kept at an average temperature of 25 °C and 50 % RH. White cardboard shields were placed on each side of the apparatus as well as between the odour source flasks and the Y-tube, to avoid any visual stimuli and potential confounding effects of visual attractiveness of baits. Ambient air entering the testing apparatus was purified with passage through activated charcoal granules (12 - 20 mesh particle size, DARCO®, Merck) and humidified when passing through a 500 ml Erlenmeyer flask that contained 200 ml deionized water that was heated by a hotplate with thermostat to a temperature of 35 °C, to maintain 65 % RH within the system. These values were constantly monitored with two loggers (Escort iMini, Product MX-HE-S-8-L, Escort Data Loggers Inc). Air flowed through the apparatus using a suction pump (Schuco-Vac 230 Aspirator, Schuco® Inc) fixed to the base of the Y-tube. The test bait/lure was placed in one of the 500 ml Erlenmeyer flasks, while the negative control (Whatmann No. 1 filter paper wetted with distilled water) was placed in the other. All baits were used within an hour after preparation for the experiment. Airflow was regulated manually, and kept at a constant rate of 100 ml/min, using flow meters (Model P with CV™ valve, 150 mm meter, Aalborg®) attached to each arm of the Y-tube. After each experimental run (i.e. after 33 replications per bait/lure placed on either the left or right side of the Y-tube) the glass Y-tube, PVC tubes, connectors and any other component used to handle wasps or baits/lures, were cleaned with an odourless, residue-free solution (Extran® MA05, Merck), rinsed in hot water and left to dry until the following day.
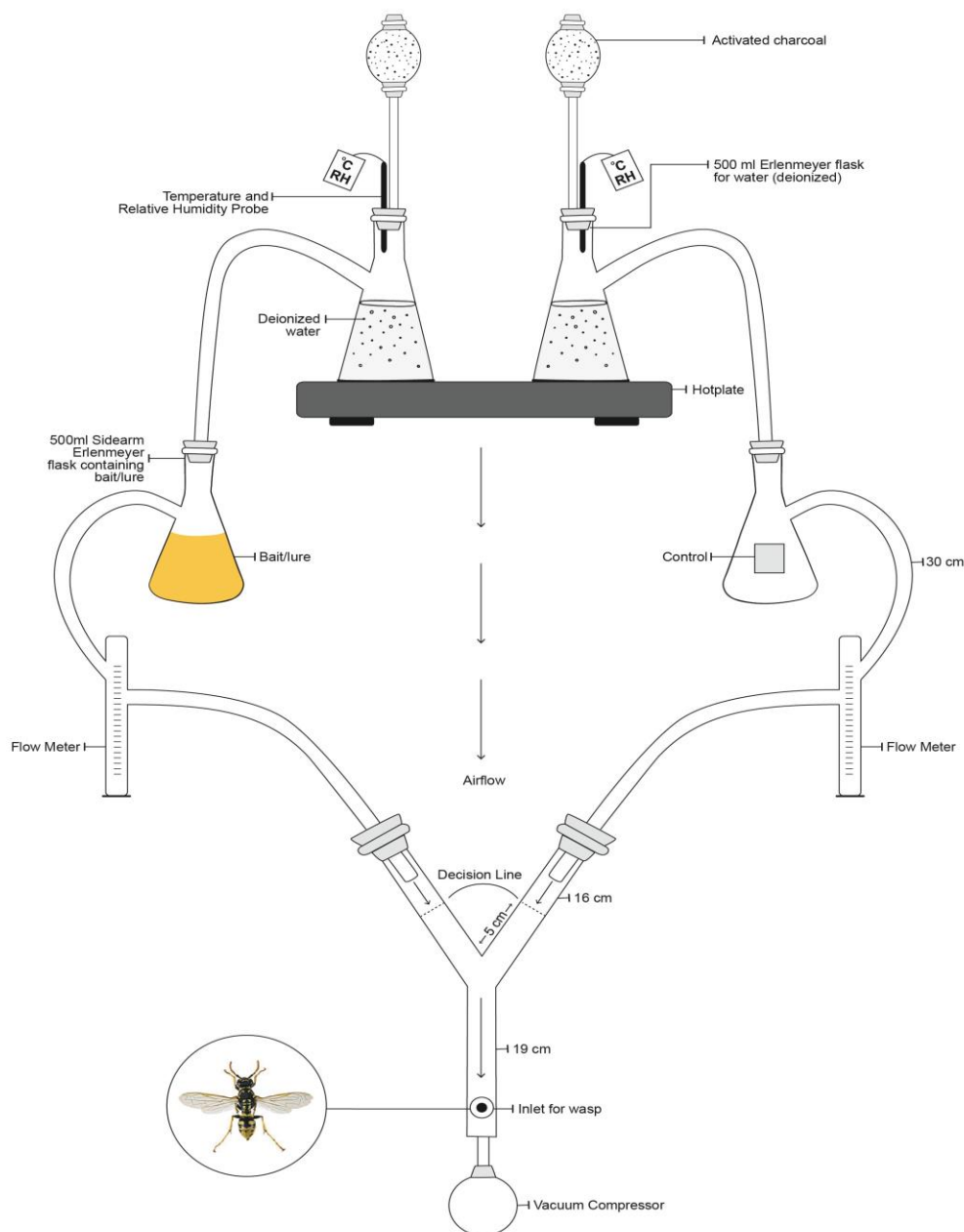
Fig. 2 The Y-tube olfactometer setup for recording behavioural choice responses of *Vespula germanica* and *Polistes dominula* wasps to a variety of baits and lures.

Behavioural bioassay

The dual-choice experiment in the olfactometer was conducted from 08:00 - 13:30 every day, as high wasp foraging activity is observed in the field throughout the morning (Edwards 1980; Richter

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2000). The entire olfactory system, containing the selected bait/lure and control, was set up and airflow switched on 10 min before *V. germanica* or *P. dominula* wasps were gently dropped one-by-one, by using forceps, through an inlet into the base of the Y-tube and each individual observed for 5 min. A choice was recorded when the wasp moved upwind, crossed the decision line of either the control arm or the bait/lure arm and remained behind that line for 30 s. The time it took the wasp to make a decision was also recorded. If the wasp remained in the common arm of the Y-tube during the 5 min observation period, it was noted as a no-choice and the wasp was removed. Altogether 33 wasps were tested in succession per day and each individual wasp was only used once. The same bait/lure was switched to the other arm the following day, to compensate for a potential position bias in the Y-tube. Therefore, in each series, a maximum of 66 wasps were tested: 33 wasps with the bait/lure on the right side of the Y-tube, and 33 wasps with the bait/lure positioned on the left side. Between each positional change and bait/lure assay, a new set of wasps and cleaned equipment were used.

Identification of chemical components in the headspace

A sample of the bait/lure that elicited the highest significant positive response in *V. germanica* (ham), was sent to the Central Analytical Facility (CAF) at Stellenbosch University where gas chromatography mass spectrometry (GC-MS) was used to identify all volatile and semi-volatile components in the headspace. Ham was also confirmed to be an attractive bait for *V. germanica* by Haupt (2014) and that is why this bait only was sent for analyses. The headspace is the space above the sample of interest in a chromatography vial where volatile components in the gas phase are present.

Data analyses

To compare the respective responses of *V. germanica* and *P. dominula* wasps to baits/lures, we assume that (p) is the probability of choosing the direction where the bait is stored. The null hypothesis (H_o) states that the probability (p) of the wasp to respond to the bait/lure is $p = 0.5$ (meaning either direction is equally possible) versus the alternative hypothesis H_1 that $p > 0.5$, meaning that the probability of choosing the right (or left) direction is bigger than 0.5. This hypothesis was tested using a binomial distribution with a correction for continuity (Kaspi 2000).

The response time of *P. dominula* and *V. germanica*, respectively, in selecting a specific bait/lure of preference, was compared with ANOVA. Residuals from the ANOVA were found not to be normally

distributed, so the non-parametric, Kruskall-Wallis test was interpreted. Multiple comparison p-values were used to identify which baits/lures differed significantly in terms of the time it took for the wasps to select a specific bait/lure. The times for selection were ranked from fastest to slowest reaction by both species of wasps.

To determine whether the presence or absence of developed ovaries in *P. dominula* and *V. germanica* wasps have an influence on the preference of the wasp for a specific bait/lure, the Chi-square goodness-of-fit test (at $p < 0.05$) was performed (MacKenzie et al. 2006).

Wasps that remained in the common arm of the Y-tube were not included in analyses to construct graphs (De Kogel et al 1999). All data were analysed using STATISTICA version 12 (Statsoft Inc. 2013).

Results

Bait/lure preference of Vespula germanica

The highest response from *V. germanica* was elicited by cooked ham compared to all other baits/lures tested (Fig. 3). A total of 21.8 % (12 wasps) of the 66 wasps tested moved towards the bait ($p = 0.014$). Only 8.1 % (3 wasps) moved toward the control arm (Table 3). Therefore, the H_0 is rejected, because the probability of the wasp moving toward the arm containing cooked ham, instead of the control, is higher. *V. germanica* wasps showed a similar attraction to beer and molasses - 18.2 % of all wasps that responded in favour of a lure/bait were attracted to it. The lowest attraction was toward Minute Maid[®], which acted more as a repellant, since 18.9 % wasps (7 wasps, $p = 0.008$) moved towards the control arm and none towards the bait/lure. Therefore, the H_0 that states that there is equal probability of the wasp to move toward either the control or Minute Maid[®], is rejected. It is more probable that the wasp will be repelled by Minute Maid[®] than attracted to it. *Vespula germanica* wasps also seemed to be slightly repelled by agitated *V. germanica* wasps - 18.9 % (7 wasps, $p = 0.196$) wasps moved into the clean control air stream, away from the arm linked to the flask containing agitated wasps (Appendix A). However, the result was not significant and therefore it is equally probable for the wasp to move away or towards agitated wasps.
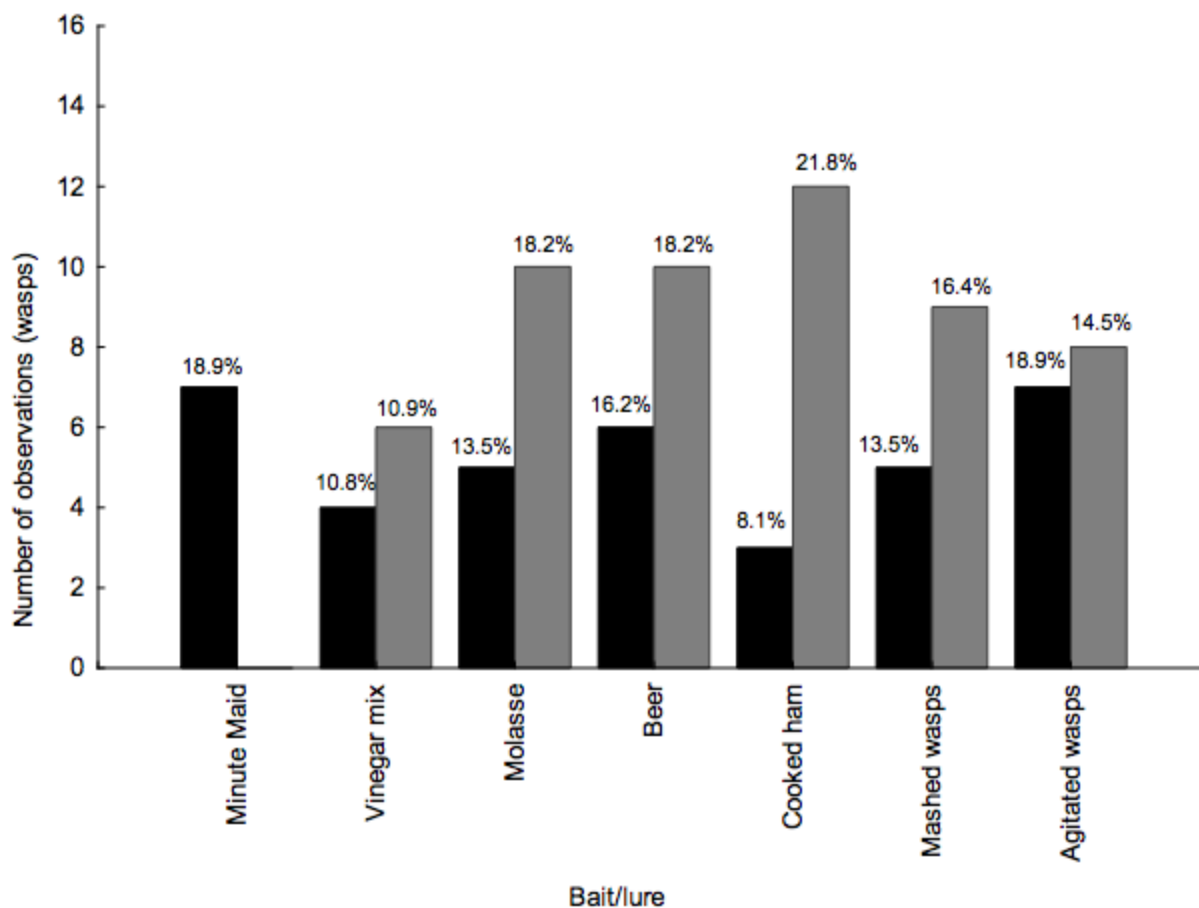
Fig. 3 Responses of *Vespula germanica* females in the Y-tube to seven different baits/lures (n = 55) and the control (n = 37). Columns indicate the mean percentages of wasps, out of all responsive wasps, that moved toward either the Y-tube arm containing the bait/lure (▮) or the control (▮).

Even though differences were observed among the preferences for baits/lures tested, the majority of wasps were indecisive and remained in the common arm of the olfactometer for the duration of the 5 min observational period (Appendix A), which minimized the sample size that were used in analyses.

Table 3 *Vespula germanica* preference that were significant for baits/lures versus control, tested in a dual choice olfactometer assay (n = 66 wasps for each bait/lure, unless stated otherwise).

Bait/lure	Number of wasps recorded			P-value (excluding indecisive wasps)
	Control arm	Indecisive	Bait/lure arm	
Minute Maid®	7	59	0	0.008
Cooked ham	3	51	12	0.014

Chemical components present at highest concentrations in the headspace

Cooked sandwich ham prompted the highest response in *V. germanica* foragers, and therefore a ham sample was sent for analysis using gas chromatography mass spectrometry (GC-MS). The compound present in the highest concentration was 3-methyl-1-butanol, which exhibits an alcoholic odour (Budavari 1989), followed by 3-hydroxy-2-butanone (acetion), which has a buttery odour (Toldrá et al. 2015).

Bait/lure preference of Polistes dominula

Polistes dominula females showed the highest response toward *P. dominula* nests, with 13.1 % (29 wasps, p = 0.013) wasps that responded to a bait/lure, moved toward the arm linked to the flask containing a nest (Fig. 4 and Table 4). Therefore the H_0 is rejected that states that the probability of the wasp to move towards either the control or *Polistes* nest is equal. Molasses elicited the second highest positive response from wasps, with 9.0 % (20 wasps, p = 0.053) attracted to it and 4.8 % (12 wasps) moving into the control arm, although it was not significant (Appendix B). The FCM lure elicited the most negative response from *P. dominula* wasps. Only 3.2 % (7 wasps, p = 0.004) wasps moved toward the flask containing the lure. FF lure repelled 10.0 % (25 wasps, p = 0.005) of all wasps that moved away from the respectively baits/lures. Therefore, there is a significant chance of wasps moving toward the control arm. Mountain Dew[®] also significantly repelled wasps (26 wasps, p = 0.009) and only 4.5 % (12 wasps) moved toward it.
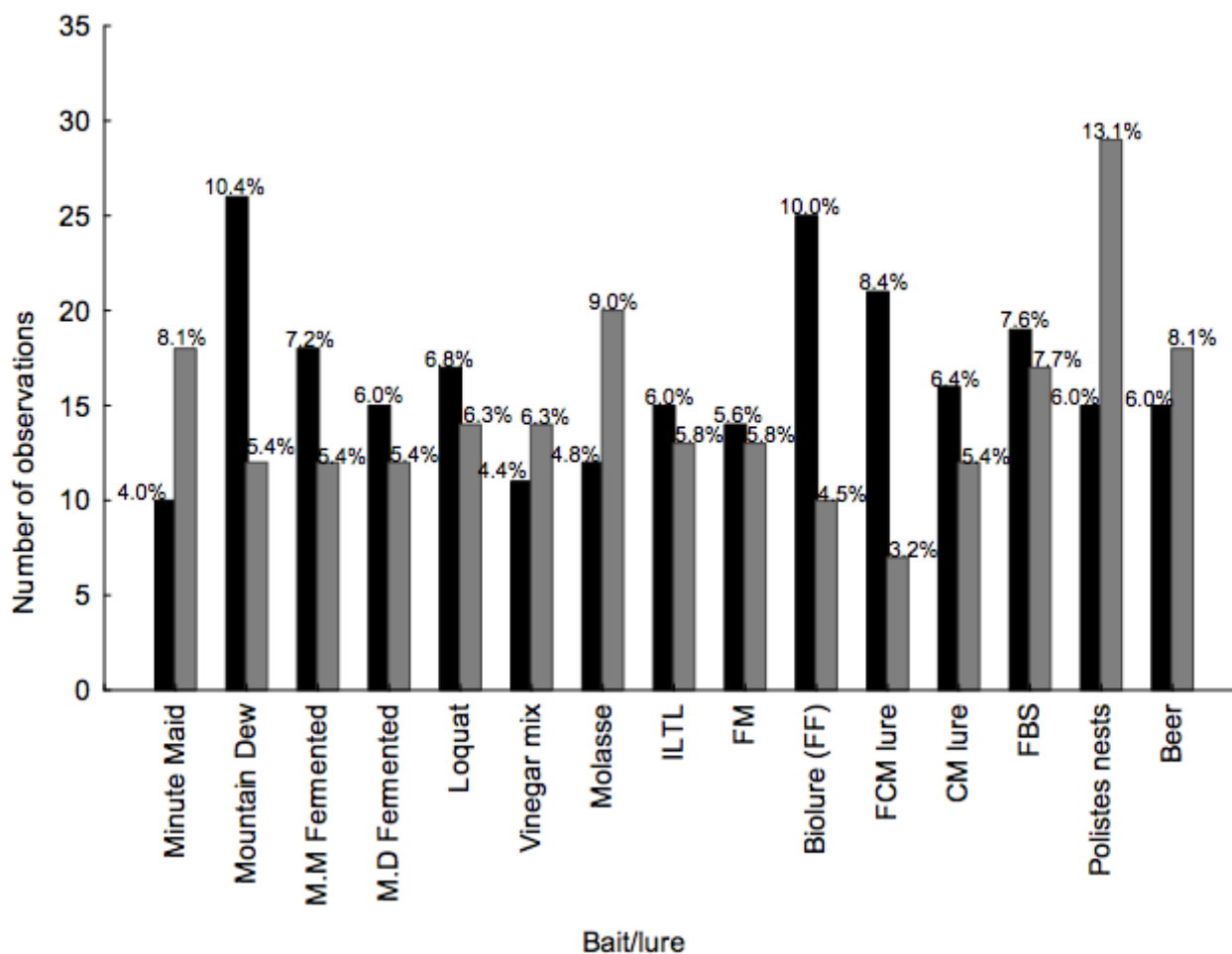
Fig. 4 Responses of *Polistes dominula* females in the Y-tube to 15 different baits/lures (n = 221) and the control (n = 249). Columns indicate the mean percentages of wasps, out of all responsive wasps, that moved towards either the Y-tube arm containing the bait/lure (▬) or the control (▬).

The high number of indecisive *P. dominula* wasps (Appendix B) compared to responsive wasps, reiterates findings in this study where *V. germanica* wasps were used in dual-choice olfactometer assays (Appendix A). However, a higher proportion of *P. dominula* wasps moved towards either the control or bait/lure arm of the olfactometer, compared to *V. germanica* wasps.

Table 4 *Polistes dominula* preference that were significant for baits/lures versus control, tested in a dual choice olfactometer assay (n = 66 wasps for each bait/lure).

Baits/Lures	Number of wasps recorded			P-value (excluding indecisive wasps)
	Control arm	Indecisive	Odour arm	
Minute Maid®	10	38	18	0.049
Mountain Dew®	26	28	12	0.009
Biolure FF	25	31	10	0.005
FCM lure	21	38	7	0.004
Polistes dominula nests	15	22	29	0.013

Response time of Vespula germanica *in selecting a preferred bait/lure*

There were significant differences (Fig. 5) in the reaction time of wasps towards the various baits/lures tested ($H_{(5, 55)}$ = 17.447, p = 0.0037). There were significant differences in the time (Table 5) it took *V. germanica* wasps to make a decision in the presence of agitated wasps, compared to the presence of the vinegar mix (p = 0.004) and molasses (p = 0.01). As noted earlier in Fig. 3, more wasps moved towards the agitated wasps. Wasps had the quickest positive reaction toward the vinegar mix (0.55 ± 0.65 min ± 1.33). However, there were no significant differences between the time it took wasps to select the vinegar mix compared to the arms linked to flasks containing molasses, beer, cooked ham and mashed wasps (Fig. 5 and Appendix C).
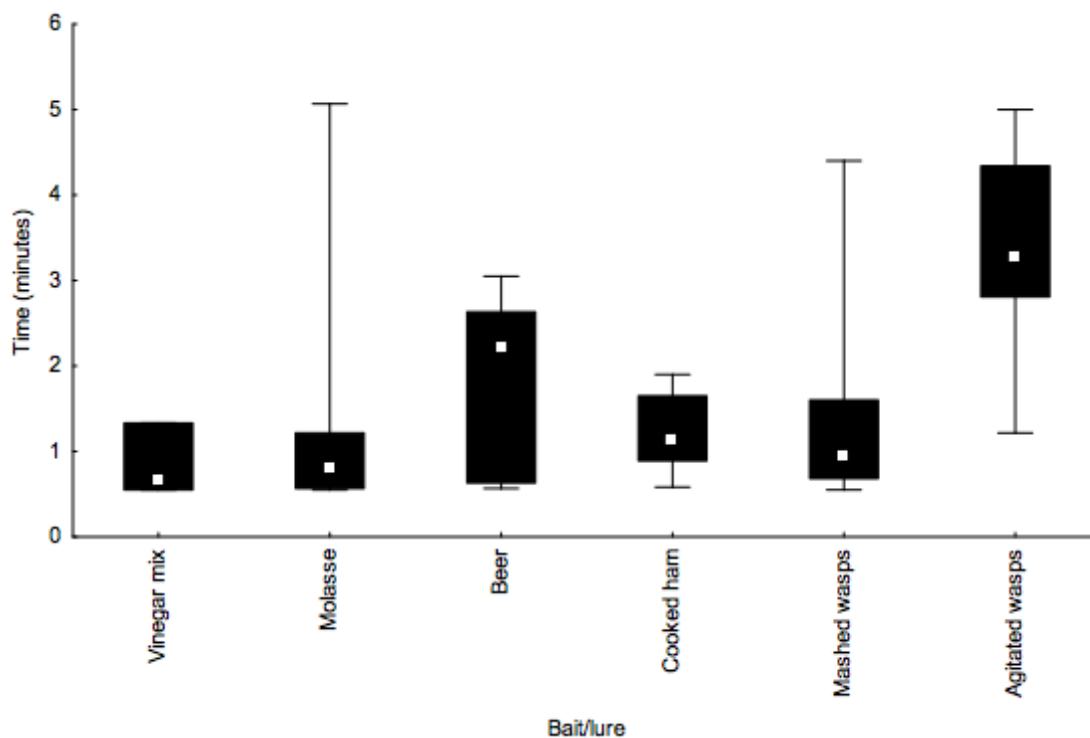
Fig. 5 Comparison between the variable times it took *Vespula germanica* female wasps to show a preference for seven different baits/lures. No wasps were attracted to Minute Maid® and therefore this bait/lure is not included on the graph. Box and whisker plots include the median, the min/max, and the 25 - 75 % quartiles.

Table 5 The significant median response times and 25 - 75 % quartiles (Q) of *Vespula germanica* wasps toward the presence of various baits/lures and the control. Times are ranked from fastest to slowest wasp reaction, either in movement towards the bait/lure or towards the control.

Bait/lure	n	Rank	Time in minutes (median)	Time (Q25, Q75)	Preference
Vinegar mix	6	1	0.65	0.55, 1.33	Bait/lure
Molasse	10	2	0.81	0.57, 1.22	Bait/lure
Agitated wasps	8	6	3.28	2.81, 4.34	Bait/lure

Response time of Polistes dominula *in selecting a preferred bait/lure*

There were no significant differences (Appendix D) among the times it took to elicit a response from *P. dominula* female wasps for any of the various baits/lures tested ($H_{(14, 221)} = 15.553$, $p = 0.341$). Wasp

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reaction to the presence of beer was the quickest, where wasps moved towards the arm emitting beer aromas after 0.60 ± 0.86 min ± 1.90. The quickest reaction (0.68 ± 1.52 min ± 1.90) of wasps moving away from a bait/lure towards the control arm, was with the FM (fermented sweet molasses) lure. The slowest reaction in terms of choosing to move was observed with the presence of FBS (fermented brown sugar). Wasps moved away from the FBS aromas toward the control arm after 1.95 ± 2.62 min ± 3.90 (Fig. 6 and Appendix D).
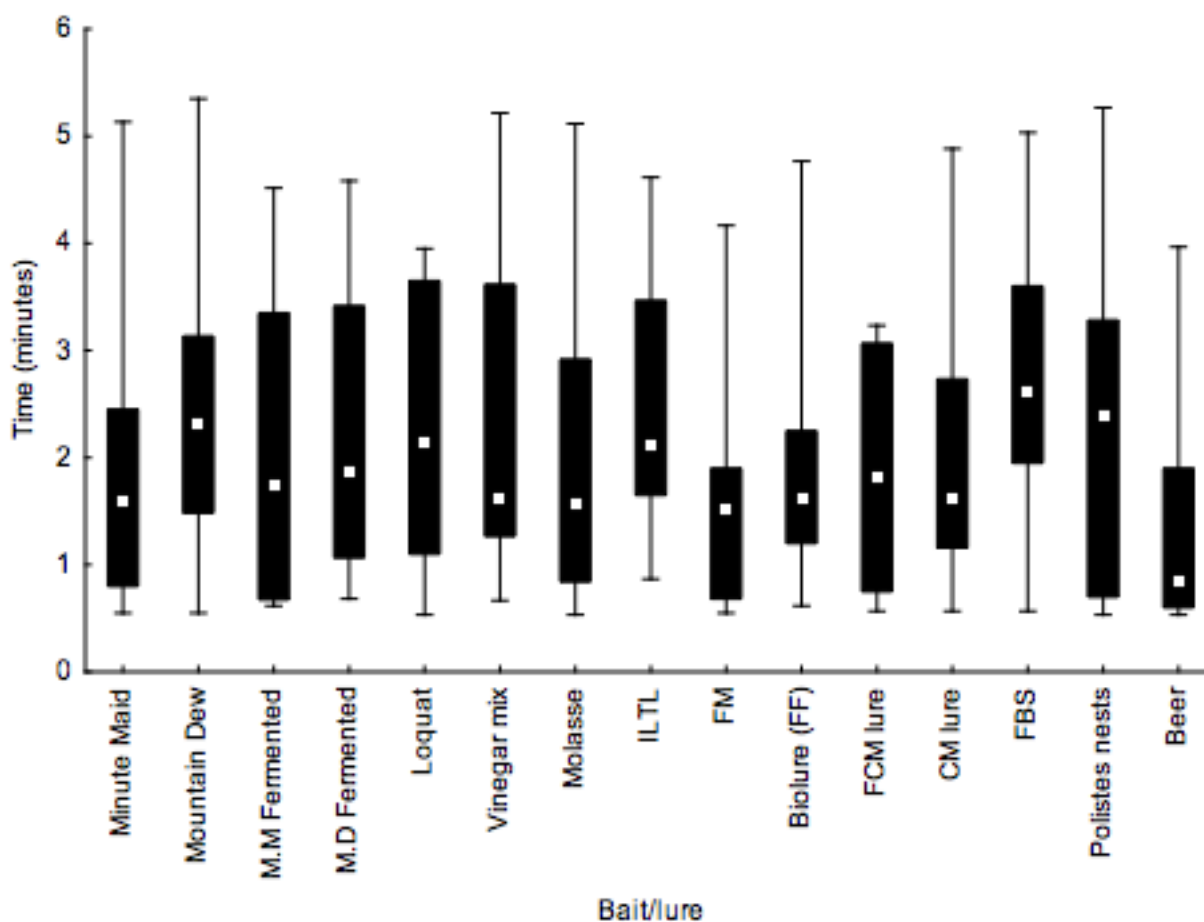
Fig. 6 Comparison between the variable times it took *Polistes dominula* female wasps to show a preference for fifteen different baits/lures. Box and whisker plots include the median, the min/max, and the 25 - 75 % quartiles.

Even though *P. dominula* wasps preferred the olfactory characteristics of a *Polistes* nest significantly more (Fig. 4) than any of the other baits/lures tested, the time it took the wasp to make a decision to

move towards the arm connected to the flask containing the nest, was quite slow and ranked second slowest (0.70 ± 2.40 min ± 3.28) out of all the baits/lures (Appendix D).

Comparison in the response of Vespula germanica *female wasps with developed ovaries versus undeveloped ovaries to selected baits/lures*

The null hypothesis which states that the proportion of wasps attracted to baits/lures are equal and independent from whether wasps have developed or undeveloped ovaries, is rejected ($p = 0.029$).

From results obtained, a significant difference in preference of *V. germanica* wasps with developed and undeveloped ovaries for specific baits/lures was observed. Where 26.09 % of all wasps with undeveloped ovaries preferred cooked ham (Fig. 7), none of the wasps with developed ovaries did. Instead, out of all the wasps with developed ovaries that reacted positively toward a bait/lure, 44.4 % (4 wasps) moved towards the arm connected to the flask containing agitated wasps, which was the highest positive response obtained for all baits/lures tested. The lowest percentage wasps with undeveloped ovaries (8.7 %) reacted positively toward the agitated wasps. It should be noted that the total number of *V. germanica* wasps with developed ovaries that were caught for these experiments, was very low (n = 41) compared to the total number of wasps with undeveloped ovaries that were caught (n = 417) and therefore comparisons and conclusions should be drawn with caution.
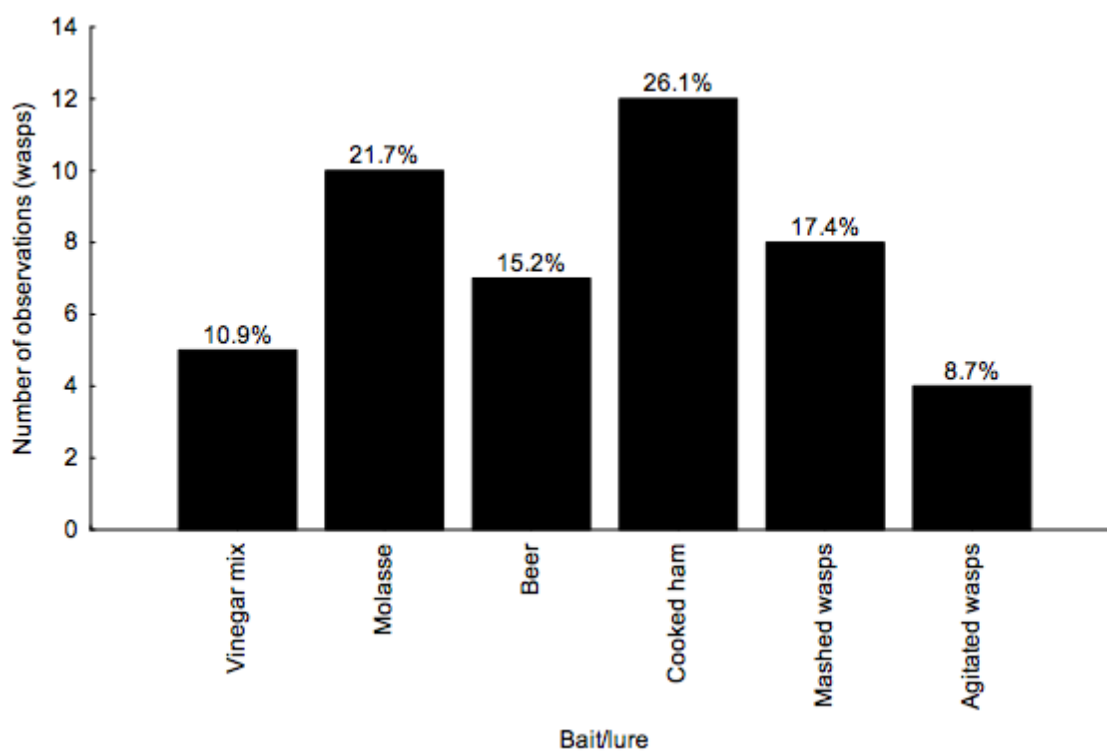
Fig. 7 The percentage positive response of female *Vespula germanica* wasps with undeveloped ovaries in reaction to the presence of the respective baits/lures in a Y-tube olfactometer setup. No wasps were attracted to Minute Maid® and therefore this bait/lure is not included on the graph.

Comparison in the response of Polistes dominula *female wasps with developed ovaries versus undeveloped ovaries to selected baits/lures.*

The null hypothesis is rejected (p = 0.004), which states that the proportion of wasp attraction to baits/lures is equal and independent from whether wasps have developed or undeveloped ovaries.

Similar to what was observed in bait/lure preference of *V. germanica* wasps with developed and undeveloped ovaries, preferences of P. *dominula* wasps with developed ovaries (n = 734) and undeveloped ovaries (n = 185) also differed (Fig. 8 and 9). Most (18.6 %) wasps with undeveloped ovaries preferred molasses above any other baits/lures, whereas most wasps with developed ovaries reacted favourably toward *Polistes* nests (13.6 %). The vinegar mix proved to be the second-most attractive bait/lure (16.3 %) for wasps with undeveloped ovaries. However, for wasps with developed ovaries, it was one of the least attractive lures (4 %), together with the FF and FCM lures. Loquat and beer, both baits/lures that wasps with developed ovaries reacted to favourably (8 % and 10.2 %,

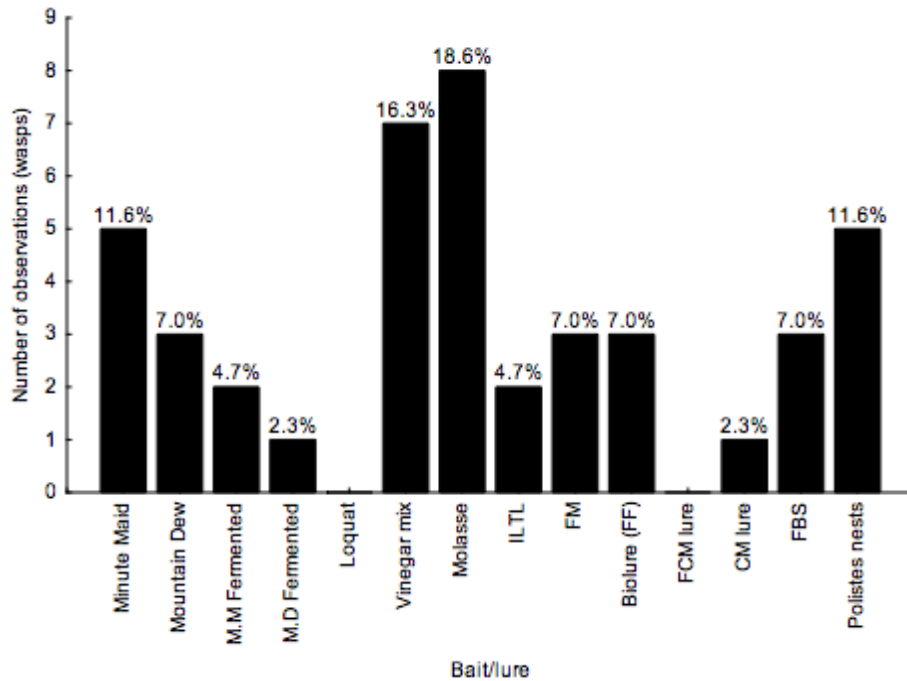respectively), did not elicit the same response in wasps with undeveloped ovaries. In fact, none were attracted to it.

Fig. 8 The percentage positive response of *Polistes dominula* wasps with undeveloped ovaries in reaction to the presence of respective baits/lures in a Y-tube olfactometer setup. No unmated wasps were attracted to beer and therefore the bar for this bait/lure is not on the graph.
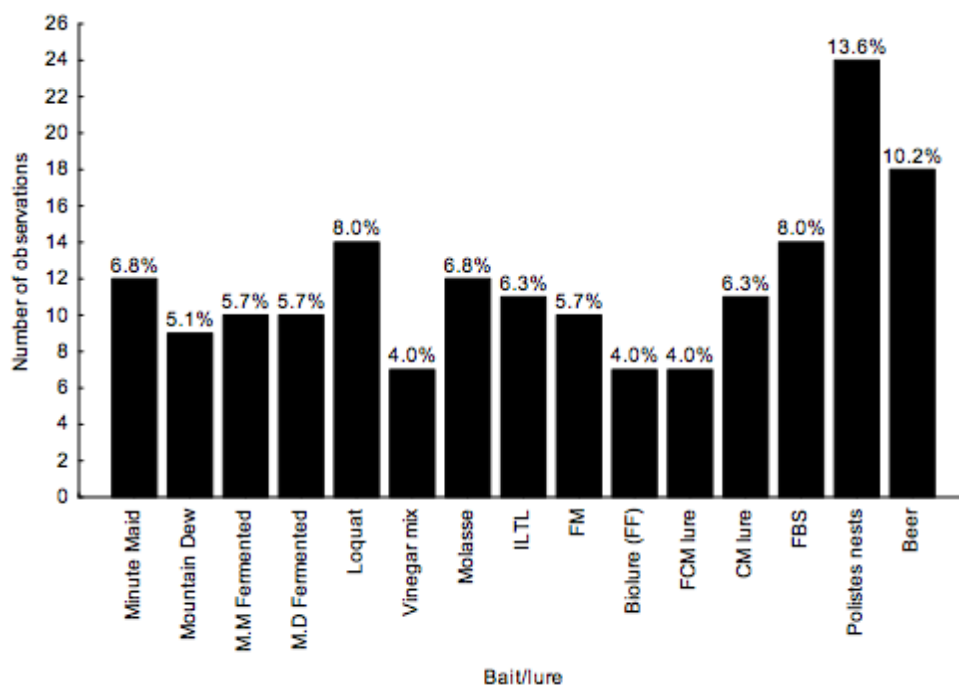
Fig. 9 The percentage positive response of female *Polistes dominula* wasps with developed ovaries in reaction to the presence of various baits/lures in a Y-tube olfactometer setup.

Discussion

Bait/lure preferences

In an effort to monitor and control the two invasive wasps, *P. dominula* and *V. germanica*, in the Western Cape of South Africa, the first objective was to determine the response to baits/lures of field collected foragers of both *V. germanica* and P. *dominula*. Currently, no implemented monitoring or management plan exists in South Africa in which specific baits/lures play a role in reducing population numbers and enabling detection and monitoring of invasive wasp populations (Tribe and Richardson 1994; Eardley et al. 2009; Giliomee 2011; Benadé et al. 2014; Haupt 2014). An effective bait/lure would improve the process of gathering information on wasp distribution and population dynamics, and reduce reliance on the public as the only information source. Public awareness campaigns are expensive and are at this stage only broadcast within certain areas where recent rough scale distribution analyses have shown wasps to be present (Benadé et al. 2014; Haupt 2014).

The local abundance of *V. germanica*, based on personal in-field observations and recent research by Haupt (2014)*,* is lower compared to other areas in the world that have been invaded. In countries such

as North America, New Zealand, Tasmania and Australia, regulations had to be implemented in an effort to avoid or circumvent direct human contact with social wasps at picnic sites, holiday resorts and schools (Spradbery and Dvořák 2010). In the case of *P. dominula*, the population density might well be comparable to other invaded countries, however, the level of wasp density for both *V. germanica* and *P. dominula* in South Africa, have not, yet, necessitated such measures as mentioned above, which might suggest that the population densities are still lower locally.

This prompted a decision to test the response of wasp foragers under controlled laboratory conditions using a Y-tube olfactory bioassay, rather than trapping in the field. Many baits/lures can be tested within a short period in a laboratory, and the possible influence of low population numbers on results (false negatives), if field trapping was conducted, is eliminated. This might explain, in part, the inconsistent results obtained by Haupt (2014), who conducted field trials over two consecutive years. Baits/lures for *V. germanica* were tested in and around the Stellenbosch area, and fewer catches using the same baits/lures the following year (February - March 2014) were obtained compared to the previous year (March - April 2013).

In this study, significant differences were obtained between the respective preferences of *P. dominula* and *V. germanica* wasps for specific baits/lures. Cooked ham was the bait that elicited the highest positive response in *V. germanica* wasps. As scavengers they are attracted to meat and sugar-based baits. Both of these substances have been used in traps for control of *Vespula vulgaris* (Linnaeus), *V. germanica* and *V. pensylvanica* (Saussure), with varying degrees of success (Wagner and Reierson 1969; Perrot 1975; Edwards 1977, 1980; Dymock et al. 1991; Spurr 1996; Spurr et al. 1996; Landolt 1998). Various volatile chemicals associated with protein-based baits/lures have shown to elicit high response in *V. germanica* (Reid and McDonald 1986; Spurr 1995; Harris and Etheridge 2001; Haupt 2014; Unelius et al. 2014). Locally, promising results were obtained using smoked ham to attract *V. germanica* foragers to traps (Haupt 2014). From the results obtained in the GC-MS analysis, where the chemical composition of the headspace volatiles of cooked sandwich ham was determined, the compound present in the highest concentration (3-methyl-1-butanol) have also been shown by Brown et al. (2014) to be one of the components that *V. vulgaris* are highly attracted to in New Zealand. El-Sayed et al. (2005) also noted it to be one of the compounds isolated in the headspace of fermented baits that certain Lepidoptera species are attracted to, such as port wine and molasses.

Spurr (1995) field tested several protein baits in New Zealand and found both *V. germanica* and *V. vulgaris* mostly attracted to raw, lean meat and fish species with high oil content, such as salmon. In the same study, *V. germanica* and *V. vulgaris* were attracted to sardine cat-food, and the attraction

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increased from January to March, when wasp population numbers peaked. Less promising results were obtained when Haupt (2014) conducted preliminary field trials under local conditions, using tinned cat food. Other proteinaceous sources that have shown to attract *Vespidae* are: volatiles from green-lipped mussels, tested in New Zealand (Brown et al. 2014; Unelius et al. 2014); minced beef in Argentina (Sackmann and Corley 2007); volatiles from fly attractants in Brazil (Richter and Jeanne 1985); fish in Argentina (Pereira et al. 2013); wallaby meat in Tasmania (Statham 2001); freeze-dried kangaroo and canned chicken meat in Australia (Wood et al. 2006); horsemeat in Eastern North America (Ross et al. 1984); and beef in Argentina (Sackman and Corley 2007).

In the UK it has been noted by Edwards (1980) that wasps readily prefer protein baits where natural food such as flies and dead animals are abundant, but when protein baits were applied near sweet factories and bakeries, wasps were not attracted to it. In this study, all wasps were collected from a relatively small area in and around Stellenbosch and, therefore, the specific wasp population might be accustomed to protein baits. Especially since the wasp population density of *V. germanica* seem to be lower compared to other countries, such as New Zealand and Australia (Barlow et al. 1996; Haupt 2014), a possible excess of protein sources are available for the wasps to feed on in the field and they could, therefore, be conditioned to react preferentially towards volatiles associated with it. Yet, *V. germanica* populations in and around Cape Town might not necessarily exhibit similar preferences to their Stellenbosch counterparts for meat-based baits. The immediate surrounding environment influences food preferences of wasps, and baits/lures found to be effective in one region will first have to be field-tested in the other to confirm efficacy. Additionally, the fact that this experiment was conducted using only a single season's wasps should be taken into consideration before utilizing protein-rich baits as part of a monitoring or control programme. The trial should first be repeated during the same time of year to confirm the observed preferences of *V. germanica* populations from Stellenbosch. Furthermore, various meat-based baits should be tested in a Y-tube setup, such as minced beef that Haupt (2014) has demonstrated to be attractive to *V. germanica* populations from Stellenbosch.

Several problems are unfortunately associated with the use of fresh meat baits in the field. They need to be replaced regularly to remain attractive (Ross et al. 1984; Reid and MacDonald 1986; Spurr 1995; Wood et al. 2006) and can therefore be expensive (Landolt 1998). The low specificity of meat-based baits make it attractive to non-target species such as birds and ants (D'Adamo et al. 2001). Edwards (1980) noted that it is suspected that wasps in urban settings prefer carbohydrates to proteins (Spurr

1996), which further complicates bait/lure selection. The focus of future research should therefore include other forms of baits/lures as well, apart from meat-based baits. Since limited research has been conducted on bait/lure selection for *V. germanica* in South Africa, the addition of humectants or synthetic food attractants to meat-based baits, could in the interim potentially improve bait field-life without compromising its attractiveness (Spurr 1995; Landolt 1998).

Of all baits/lures tested for *V. germanica*, Minute Maid[®] was the only one that showed no attraction and the highest and significant level of repellence. This contradicts findings by Wegner and Jordan (2005) in Ohio, Northern America, where the attractiveness of citrus-based sodas, including Minute Maid[®], was compared to the synthetic lure used by Landolt et al. (2000) which consisted of isobutanol and acetic acid, and was found to perform better than the synthetic lure. Several species of *Vespula*, *Polistes* and *Dolichovespula* were trapped in high numbers using Minute Maid[®]. This disparity may be attributed to the local *V. germanica* population's potential nutritional preference for protein food sources. Local wasp colonies may also have been at a different developmental phase and therefore brood requirements would have differed (Sackmann and Corley 2007). However, the fact that the highest number of wasps remained in the common arm of the Y-tube and no wasps were attracted to Minute Maid[®], might suggest the presence of a compound in the soda that *V. germanica* are repelled by. In identifying such a compound, its properties could potentially be utilized to an advantage in areas where wasp presence needs to be reduced, such as public congregation spaces or private homes.

In testing the attractiveness of various baits/lures for *P. dominula*, it was not unexpected to find wasps reacting most significantly favourably towards their own nest in the olfactory Y-tube setup. It is known that wasps mark their nests with chemical substances and use these substances to recruit colony members (Field et al. 2000; Claudia et al. 2010) and mediate recognition of nests and nest mates. It was also the only odour cue tested, where the numbers of indecisive wasps were less than the number of wasps that decisively chose either the control or bait/lure arm of the Y-tube. Even though field collected wasps from various other nests will not be as attracted to the nest from this specific colony of wasps, the result confirms that the chemical substances (pheromones) which social wasps use to communicate, are powerful tools that should be harnessed for control efforts. Pheromones are used for the control of many other species, especially pestiferous moth species (El-Sayed 2013; Lester et al. 2013). Due to the behaviourally complex nature of social insects (Welter et al. 2005; El-Sayed et al. 2006, El-Sayed et al. 2009), limited research has been conducted in utilizing pheromones for wasp control purposes (Ward 2013). Great opportunity exists to better explore this subject, especially in a

local context, where the *V. germanica* population density are low to moderate (Haupt 2014). The use of pheromones to manage pest populations, are most effective at lower densities (Ward 2013). Two pheromones are emphasized by Claudia et al. (2010) in terms of its potential as control measures, namely queen pheromones and nest-mate recognition pheromones. Due to the specificity of pheromones, identifying an effective species specific chemical compound(s) that elicits the desired reaction for successful wasp management, will reduce non-target impacts on beneficial species. Pheromones can also be synthetically produced, which would be more practical and last longer in the field, compared to fresh baits/lures (Landolt 1998).

Unfermented molasses was more attractive to *P. dominula* than fermented molasses. *P. dominula* foragers also preferred fresh Minute Maid® over the fermented sample, and more foragers were repelled by olfactory cues from FBS than attracted to it. This result is interesting, because to date a lot of focus on chemical lure development for wasps has been on compounds that are structurally similar to chemical products that mimic rotting or fermenting fruit and sugary substances (Landolt et al. 2000; Day and Jeanne 2001; Dvořák and Landolt 2006; Dvořák 2007). From results obtained, one would be tempted to deduce that local *P. dominula* foragers prefer olfactory cues of fresh products. However, caution should be exercised in making such an absolute conclusion, because the possibility that some of the specific compounds that are usually found in fermenting fruit juices (Landolt et al. 2000), do exist, were not produced in the fermented products tested, since the headspace volatile composition of all baits/lures tested, were not analysed. Many factors can play a role in the attractiveness of specific compounds to wasps, of which temperature and synergy between chemicals are important (Landolt et al. 2007).

FCM lure, Biolure® FF and Mountain Dew® elicited the highest significant levels of repellency of *P. dominula.* This result contradicts findings by Wegner and Jordan (2005), where Mountain Dew®-baited traps were the most attractive baits for *Vespula*, *Polistes* and *Dolichovespula* species combined. The sugar content of Mountain Dew® is 10 % and some other listed ingredients include orange juice concentrate, citric acid, sodium citrate, gum Arabic and erythorbic acid. Results of this study suggest that one or more of the chemical compounds present in this soda drink, are disagreeable to presumably indigenous *P. dominula* foragers. If so, the exact component responsible for deterrence should be identified in future studies. This reaction may also, as previously stated, be attributed to different food preferences of local *P. dominula* wasps.

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Biolure® FF, an attractant used to monitor *Ceratitis* spp. in local fruit orchards, was included in this trial, because in a study conducted by Richter and Jeanne (1985), wasps were found to be attracted to a fly lure, which contained heptyl or octyl butanoate, amongst other ingredients. These compounds have found to be attractive to wasps (El-Sayed et al. 2009). The exact composition of Biolure® FF is not known and even though the active ingredients listed in this lure do not include heptyl or octyl butanoate, the lure was still included in the trial to observe whether it would prompt a response from the wasps, negative or positive. Of all the formulated, commercially available lures tested (FCM, CM and FF), the proportion of wasps that moved towards the control arm of the Y-tube, was higher than those that moved towards the lure-containing arm. In addition, the FCM lure was also the least attractive lure of all lures tested for *P. dominula*. Therefore, the presence of these lures in orchards might, unwittingly, deter *P. dominula* wasps. However, monitoring experiments will first have to be conducted *in situ* to confirm this hypothesis.

Response time of wasps in selecting a preferred bait/lure

The time it took *V. germanica* females to respond to olfactory cues of the various baits/lures, differed from the order of bait/lure preference. Wasps reacted fastest in the presence of the vinegar mixture, even though cooked ham was the most preferred bait. This can be attributed to highly volatile components present in vinegar, of which acetic acid is the most abundant (Landolt et al. 2014). A small proportion of the vinegar mixture also included another highly volatile component, namely ethanol/alcohol (EtOH). In a Y-tube olfactometer study conducted by Landolt et al. (2014), *Polistes metricus* (Say) and *Polistes bellicosus* (Cresson), were attracted to wine, but not vinegar, but when traps containing a blend of wine and vinegar were placed in the field, many of these two wasp species were captured. It is not uncommon for wasps to react differently in the field in response to baits/lures, than what is observed in a laboratory. In the field, they may be exposed to multiple choices simultaneously, whereas in the laboratory, in this case, they were exposed to odours one at a time. The quick reaction to the vinegar mixture could in part be attributed to its higher volatility, which makes it easier for the wasps to pick up the odour under field conditions. Therefore, choosing baits/lures that are highly attractive and that contain highly volatile components, might make it more effective under field conditions. Alternatively, highly volatile components can be combined with protein-based baits, such as ham in this case, which seems to consist of less volatile compounds.

The slow reaction of *V. germanica* observed to agitated wasps, could be attributed to confusion. As stated earlier, pheromones are highly effective communication substances. When wasps are aggravated,

they release an alarm pheromone (Moritz and Bürgin 1987; Mashaly et al. 2012). Within the closed space of the closed Y-tube system, the presence of this pheromone might have saturated the air and caused confusion.

As with *V. germanica*, the time it took *P. dominula* to respond to a specific bait/lure, did not match the order of bait/lure preference. Wasps moved quickest toward the bait-arm of the Y-tube when it contained beer. The finding by Landolt et al. (2014), that another *Polistes* sp., *P. bellicosus*, is attracted to ethanol, both under field conditions and in a Y-tube setup, supports this result. The beer used in this study had an alcohol content of 3.8 % vol. It could be possible that other volatiles specifically related to beer are an additional source of attraction, since beer has been previously used in baits to trap wasps (Dvořák 2007) and was the third most preferred bait/lure, together with Minute Maid®, for *P. dominula* wasps in this trial.

Polistes nests were, as expected, the most attractive substance tested in the Y-tube for *P. dominula* wasps, but a delayed reaction was observed compared to the other baits/lures. Aside from the slow reaction time to FBS, it took wasps the longest time on average to move into the arm containing the nest. This can potentially be attributed to the low volatility and low concentration of nest pheromones present on the nest and live larvae. The nest in the flask was also small (6.47 g). However, due to the effectiveness in luring most of the wasps tested in the tube towards the nest, further research should focus on isolating and identifying the compounds present on *Polistes* nests, so that synthetic pheromones can be produced where concentration and volatility can be modified to increase efficacy. Unlike with *V. germanica*, there were no significant differences in the time it took *P. dominula* wasps to react to the various baits/lures.

Comparison in the response of female wasps with developed versus undeveloped ovaries to selected baits/lures

The experimental trial was conducted over a period of 6 months and therefore, out of practical necessity, both females with developed and undeveloped ovaries were caught in the field during wasp collection. Due to potential differences in responses to olfactory cues of wasps with developed and undeveloped ovaries, a distinction was made between the two groups to determine whether the difference in reproductive state influences bait/lure preference. Only female wasps were included, because they are the primary foragers in the caste system. The target in this study was forager wasps, where the aim of developing a lure is to employ it to reduce wasp population levels and this is most

effectively achieved by mobilizing forager wasps as vectors of baits/lures that contain toxins (Ward 2013).

From the results obtained, there were significant differences between the preferences of female *V. germanica* wasps with developed and undeveloped ovaries for certain baits/lures. However, further comparisons and conclusions are limited by the very low total number of *V. germanica* females with developed (n = 41) versus undeveloped ovaries (n = 417) that were caught during this experiment. Catches for *V. germanica* were conducted in the summer and autumn (January - April 2015) when queen wasps remain on newly built nests and sterile females forage for food. In an effort to obtain a more detailed reflection of differences in bait/lure preferences between *V. germanica* females with developed and undeveloped ovaries, future research will have to test equal numbers of both states of female wasps in a Y-tube setup.

In the case of *P. dominula*, mostly females with developed ovaries were caught (n = 734) compared to females with undeveloped ovaries (n = 185). Once again an imbalance between the numbers of females with developed and undeveloped ovaries occurred, even though it was less skewed compared to *V. germanica*. *Polistes dominula* wasps were caught over a longer period (from October 2014 to February 2015), when it is spring and summer. One would expect that females with undeveloped ovaries would be more abundant during these seasons. The proportion of wasps with developed ovaries caught versus undeveloped ovaries was therefore surprising. This result indicates that the annual life cycle of *P. dominula* under local conditions, might differ from what it is in its native range and would definitely be worth further investigation.

Due to the same disparity between *P. dominula* with developed and undeveloped ovaries in terms of wasp numbers, the influence on the outcome should be taken into consideration before drawing definite conclusions about bait/lure preferences. It is clear that both *P. dominula* females with developed and undeveloped ovaries responded favourably to their own nest.

It should be kept in mind that the indecisive wasps and those that chose the control, had to be eliminated from the results in order to analyse the data for specific conclusions to be drawn from the analyses. Unfortunately, throughout this experiment, a very large number of wasps, for both wasp species, remained indecisive in the Y-tube in response to baits/lures. This resulted in a low number of repetitions that could be included into statistical analyses, especially in the last assay, where the preferences of wasps with developed and undeveloped ovaries were determined. Since it is challenging, without exercising destructive sampling, to confirm the ovarian state of field-collected female wasps before the Y-tube experiment is conducted, it was not possible to know in advance exactly how many

females with developed and undeveloped ovaries would be part of the analysis. In future research the number of wasp samples should be increased. A bigger sample size will result in more pronounced significant differences between observations. Another solution would be to rear a colony of wasps in the laboratory, where reproductive stages can be regulated and closely monitored. However, laboratory reared social insects in experiments have its drawbacks as well (Jandt et al. 2015). Finally, the number of non-responsive wasps could be reduced by starving them before they are used in the Y-tube. Some olfactory Y-tube studies starved wasps beforehand (MacKenzie et al. 2006; Landolt et al. 2014). There is also the possibility that none of the baits/lures that were tested were highly effective. Testing new lures, or combining existing lures, or specific chemical compounds, should remain a research priority.

To conclude: all results allude to the preferences of *P. dominula* and *V. germanica* females for various baits and lures, under controlled conditions, of which there are no prior reports for wasp populations of these two invasive species in South Africa, apart from the study conducted by Haupt (2014) for *V. germanica*. The results do not necessarily indicate how wasps will respond in the field toward similar baits/lures. Since many of the wasps remained unresponsive in the Y-tube, it is suggested that the range of bait and lure selection be expanded, before efforts are made to optimize the best performing baits and lures of this study. When selecting alternative materials to test for attractiveness, it should be kept in mind that many other factors can also influence foraging activity and bait preference, such as the weather (Harris et al. 1991; Spurr 1995); visual stimuli (D'Adamo and Lozada 2003; Moreyra et al. 2006), season (Wegner 2003); colony developmental phase (Spurr 1996; Wood et al. 2006; D'Adamo and Lozada 2007; Sackmann and Corley 2007); wasp density and available food sources (Richter 2000; D'Adamo and Lozada 2007; Kasper et al. 2008).

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Appendix A

Table 3 *Vespula germanica* preference for baits/lures versus control, tested in a dual choice olfactometer assay (n = 66 wasps for each bait/lure, unless stated otherwise).

Bait/lure	Number of wasps recorded			P-value (excluding indecisive wasps)
	Control arm	Indecisive	Bait/lure arm	
Minute Maid$^{®}$	7	59	0	**0.008***
Vinegar mix	4	56	6	0.205
Molasses	5	51	10	0.092
Beer	6	50	10	0.122
Cooked ham	3	51	12	**0.014***
Mashed wasps (n = 62)	5	48	9	0.122
Agitated wasps	7	51	8	0.196

*Indicates significant attraction or repulsion

Appendix B

Table 4 *Polistes dominula* preference for baits/lures versus control, tested in a dual choice olfactometer assay (n = 66 wasps for each bait/lure).

Baits/Lures	Number of wasps recorded			P-value (excluding indecisive wasps)
	Control arm	Indecisive	Odour arm	
Minute Maid®	10	38	18	**0.049***
Mountain Dew®	26	28	12	**0.009***
Fermented M.M	18	36	12	0.081
Fermented M.D	15	39	12	0.130
Loquat	17	35	14	0.123
Vinegar mix	11	41	14	0.133
Molasse	12	34	20	0.053
ILTL	15	38	13	0.139
FM	14	39	13	0.149
Biolure FF	25	31	10	**0.005***
FCM lure	21	38	7	**0.004***
CM lure	16	38	12	0.113
FBS	19	30	17	0.125
Polistes nests	15	22	29	**0.013***
Beer	15	33	18	0.121

*Indicates significant attraction or repulsion

Appendix C

Table 5 All median response times and 25 - 75 % quartiles (Q) of *Vespula germanica* wasps toward the presence of various baits/lures and the control. Times are ranked from fastest to slowest wasp reaction, either in movement towards the bait/lure or towards the control.

Bait/lure	n	Rank	Time in minutes (median)	Time (Q25, Q75)	Preference
Vinegar mix	6	1	0.65	0.55, 1.33	Bait/lure
Molasse	10	2	0.81	0.57, 1.22	Bait/lure
Mashed wasps	9	3	0.95	0.68, 1.60	Bait/lure
Cooked ham	12	4	1.14	0.89, 1.65	Bait/lure
Beer	10	5	2.21	0.63, 2.63	Bait/lure
Agitated wasps	8	6	3.28	2.81, 4.34	Bait/lure

Appendix D

Table 6 The median response times and 25 – 75 % quartiles (Q) of *Polistes dominula* wasps towards the presence of various baits/lures and the control. Times are ranked from fastest to slowest wasp reaction, either in movement toward the bait/lure or toward the control.

Bait/lure	n	Rank	Time in minutes (median)	Time (Q25, Q75)	Preference
Beer	18	1	0.86	0.60, 1.90	Bait/lure
FM	13	2	1.52	0.68, 1.90	Control
Molasse	20	3	1.58	0.84, 2.92	Bait/lure
Minute Maid	18	4	1.60	0.80, 2.45	Bait/lure
Vinegar mix	14	5	1.61	1.27, 3.62	Bait/lure
Biolure (FF)	10	6	1.61	1.20, 2.25	Control
CM lure	12	7	1.61	1.16, 2.73	Control
M.M Fermented	12	8	1.73	0.68, 3.35	Control
FCM lure	7	9	1.82	0.75, 3.07	Control
M.D Fermented	12	10	1.87	1.07, 3.42	Control
ILTL	13	11	2.12	1.65, 3.47	Control
Loquat	14	12	2.13	1.10, 3.65	Control
Mountain Dew	12	13	2.32	1.48, 3.13	Control
Polistes nests	29	14	2.40	0.70, 3.28	Bait/lure
FBS	17	15	2.62	1.95, 3.60	Control

Chapter 6

Conclusions and Recommendations

This study of *Vespula germanica* and *Polistes dominula* under South African conditions mainly relied on the abundant international literature that informs the two wasp species' biology, impact, potential threats and control in their endemic ranges or in invaded countries other than South Africa. The limited and incomplete local records and research conducted in South Africa on these wasps generally made it difficult to draw definite conclusions, even when results pointed in a particular direction or showed great promise. It is understandable that few investigations into the presence of both wasp species in South Africa have been launched and limited action taken to address their expansion. The fact that their population numbers have remained relatively low since introduction, are known to fluctuate annually or over a period of a few years, and their spread have been relatively slow so far, all contribute to the lack of interest and concerted effort to control or eradicate them.

As a result of scarce and intermittent record keeping referencing the invasive wasp situation in South Africa, it was decided to firstly establish the status of *V. germanica* and *P. dominula* in the field. It was noted, that the population densities of especially *V. germanica*, were much lower than anticipated and the impression given by earlier records. It was harder to find *V. germanica* nests than those of *P. dominula*. This observation could be attributed to the nesting habits of *P. dominula* which make their nests more visible, as well as the presence of higher densities in the Stellenbosch area of this species compared to *V. germanica*. Many *V. germanica* nests were also active until much later in the winter season, whereas *P. dominula* nests were abandoned as soon as temperatures dropped. In addition, a few very large *V. germanica* nests were excavated in late winter, and when opened, contained wasps of various different life stages and sexes, suggesting that these nests were overwintering nests.

An interesting discovery was the high parasitization levels by a fly species of every single *P. dominula* nest that was used in the biocontrol field trial. On average 13 % of all cells were parasitized at any given stage of wasp nest development. This percentage might seem low, but given that only a part of the nest cells are occupied by wasp larvae/pupae throughout nest development, the difference between the number of parasitized cells and cells containing wasp larvae/pupae was quite small. The exact extent to which this parasitism contributes in reducing population numbers of *P. dominula* is unclear and fell beyond the scope of this study. It should, however, be explored in future research projects, especially since the fly and parasitic wasp species were also found to occur in *P. dominula* nests by a

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masters student working on the same project. The presence of these parasitoids in *P. dominula* nests could potentially have a meaningful impact in reducing invasive wasp population numbers. Furthermore, if more information is available on the biology of these parasitoids, the surrounding environment could be optimized to promote their survival, fecundity and parasitization of wasp nests.

There are potentially many natural enemies of *V. germanica* and *P. dominula* that remain to be discovered in their current invaded range in South Africa, but in the interim, presumably indigenous biocontrol agents that can be mass-produced in the laboratory, or are commercially available in formulated form, should and were tested for pathogenicity toward these invasive wasp species. Very promising results were obtained in the laboratory with the three presumably indigenous entomopathogenic nematode species tested as well as the one presumably indigenous, commercially produced entomopathogenic fungal species. However, disappointing results were obtained under field conditions. This result demonstrated that while the biocontrol agents may be highly pathogenic towards the wasp larvae, the time of application influences the mortality level and an effective formulation and application method is vital to the successful performance of the biocontrol agent under field conditions. Optimizing these aspects will entail much more research. A decision will have to be made on whether such research will be worth the time and financial input, or whether the focus should be on using chemical pesticides and optimizing their method of application. It would be preferable to limit the use of chemicals in populated areas as both invasive wasp species are associated with humans. If a biocontrol agent could be identified which is host specific, pathogenic, and recycles itself in the field each year, further optimization would be justifiable. It should be kept in mind that the successful management of these wasps is time-sensitive due to the continuous spread of both species.

It is believed that the lure-and-kill application method would be the most effective control strategy. Several studies have proved that targeting and trapping only foragers have no significant impact on wasp population density. This control method might be more applicable in situations where wasps need to be excluded from small areas, such as picnic sites. Ideally, to ensure the control agents reach the colony, foraging wasps should be lured to a specific point, where bait containing the biocontrol agent, or slow-acting pesticide, or both, are taken back to the nest and fed to the brood. Under these conditions, the attractiveness of the lure plays an extremely important role. Not only will it affect the level of control obtained, but it will also influence the efficacy of monitoring practices. Developing a lure that could outcompete other sources of attraction in the field is of even greater importance under local conditions, due to the lower population densities of especially *V. germanica* in comparison to some of the other invaded countries.

Literature concerning lures for social wasps, indicated that the main problems faced were that: wasps' preference for specific baits/lures changes throughout the year; many baits and lures were not host specific and attracted honeybees as well; the efficacy of lures is influenced by environmental conditions; and fresh baits/lures do not last long in the field. Therefore, various different types of lures will have to be developed and tested under local conditions to ensure that wasps can be monitored throughout the year and lured to baits containing chemical poison or the biocontrol agent. This approach would involve a cumbersome and expensive process and it is suggested that further research should focus on identifying wasp pheromones that could be used as luring compounds. Not only are pheromones highly specific in the response they elicit in certain species, but it would most probably be effective throughout the year, it can be synthetically produced, and social wasps communicate through pheromones, making them highly sensitive to it.

In this study, a Y-tube olfactometer was used to test various lures and baits for attractiveness. A large number of the both *P. dominula* and *V. germanica* wasps remained indecisive and didn't move to either the control arm or the arm that contained the lure/bait. It is suspected that using a Y-tube setup to test bait and lure attractiveness was not the ideal method to implement. The confined spaces of a Y-tube could have stressed the wasps and their handling could have exacerbated that state, leading to disorientation and behaviour that is different from that under natural conditions. It is suggested that future research should consider using other techniques to test the olfactory attractiveness of substances to wasps. Both the electroantennogram (EAG) response, which tests the responses of olfactory receptor neurons in the antennae to determine selectivity and sensitivity to olfactory cues, and placing a variety of lures in the field to compare their attractiveness, have been used in preference trials for social wasps. If wasp population densities were higher, field preference trials could have been an option, but under local conditions the chance of obtaining a false negative result is highly probable. In addition, when conducting a field preference trial, many other factors come into play that might influence the preference for a specific bait or lure, such as environmental conditions, placement of lures/baits and visual presentation of the bait or lure.

However, regardless of the efficacy and attractiveness of a specific lure or bait, the main impediment is the characteristic trait of social wasps to maintain extremely hygienic conditions within their nests. Infected nest-mates or brood will be removed from the nest as soon as these are noticed. This behaviour will prevent a sufficient quantity of biocontrol agents or poison to enter the nest to guarantee colony collapse. It would arguably be the most difficult factor to overcome if the lure-and-kill approach is pursued. Selecting a control agent that can survive on live as well as dead (nest) material might alleviate this problem. Fungal species are able to live as facultative saprophytes and the fact that fungal

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spores can be carried through air, makes them a very appealing biocontrol option. Spores can then easily be distributed through all the layers of a *V. germanica* nest and remain in the nest by surviving on nest material even when infected nest mates are continuously removed. It is suspected that the lure-and-kill technique would be more successful for use in controlling *V. germanica*, because their nests are enveloped and the temperature within the nest is regulated, which would promote spread and sporulation if an entomopathogenic fungus is used as biocontrol agent. *Polistes dominula* nests are exposed directly to environmental factors, which favour quick discard of infected nest mates or material and will complicate efforts to ensure enough biocontrol agents/poison remain in the nest to induce high mortality. This is where the efficacy of the formulated control product will play an important role in reducing loss.

One of the interesting findings that resulted from the geometric morphometrics analyses was that it seems *V. germanica* wasps have so far mainly spread by means of leading-edge dispersal throughout South Africa, which explains the slow dispersal rate. This allows time to develop and implement management strategies which could still prevent further spread to areas that are more suitable beyond the current distribution range. Even though the climatic conditions that prevail to the north in the drier Karoo area beyond the Cape Fold Mountain Belt are not ideal for *V. germanica* survival, it should be remembered that irrigated lands in agricultural production areas could drastically increase the suitability of areas for wasp establishment.

Based on the information that is currently available, it seems like *V. germanica* has struggled under local conditions to spread and reproduce to its full potential. Apart from poor climatic matching, low genetic variation might also be a responsible factor, due to potential bottlenecks that were experienced. However, further research is needed to investigate the genetic makeup of local populations to confirm this hypothesis. Combining wing geometry data with genetic data is a powerful tool to validate the results obtained in the geometric morphometric analyses and for assessing invasion pathways. Moreover, it is suggested that additional geometric morphometric analyses should be conducted, especially analyses that investigate fluctuating asymmetry (FA), which could elucidate the current fitness status of *V. germanica* populations in South Africa.

Even though research has suggested that the point of entry for *V. germanica* wasps was, and could still be, in the Cape Town area, the economical and practical feasibility of implementing regulations that would prevent further importation, needs to be researched. At this stage, the lack of quantitative data in terms of the impact of invasive wasps on agriculture, biodiversity and the nuisance and danger they pose to humans, complicate the ability to determine the associated risks if measures are not implemented to curb entry. Compared to other agriculturally important pests, it is suspected that the

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negative impact would be lower and that the various stakeholders involved would not deal with this matter with the same urgency. The current impact of these wasps may perhaps not be as much of a concern compared to other pests, but the trajectory can change rapidly as soon as they reach areas of preferred habitat along the south and east coast. Taking action at that stage might then be too late.

The first aspect that should be addressed in future studies, is the lack of basic knowledge of the biology and feeding preferences of *V. germanica* and *P. dominula* under South African conditions. There are clear differences in the lifecycle of these wasps locally compared to that in their endemic range, and these significantly influence the planning of management strategies. Generally, little information is available, specifically for *P. dominula.* Based on their spread and population density, they might pose a bigger threat than *V. germanica*. Secondly, the impact of the invasive wasps needs to be quantified to determine whether launching a management plan is defensible. Lastly, further spread beyond the perimeters of the distribution area needs to be prevented. Creating awareness and disseminating information about the wasp among the public would encourage more individuals to participate by reporting sightings and that, in turn, would help to determine the exact extent of wasp presence. Research has shown that the general consensus among landowners in the Western Cape area is that they will assist with management practices, if reliable and respected stakeholders launch a concerted management effort. It will have to be a long-term project, to ensure continued monitoring and management. All the stakeholders will also have to commit for the duration of the project.

Based on the information that has been generated by this study and previous research conducted in South Africa on the two invasive wasp species, it is concluded that at this stage it would be highly improbable to successfully eradicate *V. germanica* or *P. dominula* from South Africa. Management strategies should be implemented and optimized to reduce population densities during the wasp season and prevent further spread.