The thermal biology and thresholds of *Phytoseiulus macropilis* Banks (Acari: Phytoseiidae) and *Balaustium hernandezi* von Heyden (Acari: Erythraeidae)

By

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Abstract

Phytoseiulus macropilis Banks (Acari: Phytoseiidae) and *Balaustium hernandezi* von Heyden (Acari: Erythraeidae) have been identified as candidate augmentative biological control agents for the two-spotted spider mite, *Tetranychus urticae* Koch (Acari: Tetranychidae). The two-spotted spider mite is a significant pest of many commercial crops, including those grown in glasshouses. This study investigated the potential of both species to survive a typical northern European winter, and risk of establishment. The thermal thresholds of each species were also assessed to determine the efficacy of the predator in a horticultural system.

Through a combination of laboratory and field trials, *P. macropilis* was shown to present a low risk of establishment in northern Europe. Survival of winter field exposures was limited to three weeks, and the mite did not demonstrate the ability to enter a diapause state. Similarly, the lower thermal activity thresholds allowed movement of the mite at temperatures where *T. urticae* became immobile, but were not so low as to pose a threat of dispersal in a northern European winter. The predatory ability of *P. macropilis* on tomato and French bean leaf surfaces was investigated, and found to be similar to the current leading market predator of *T. urticae, Phytoseiulus persimilis* Athias-Henriot (Acari: Phytoseiidae).

In contrast, *B. hernandezi* demonstrated a high tolerance of northern European winter temperatures, surviving in the field for over four months. *Balautium hernandezi* was able to move at temperatures where *T. urticae* became immobile, however, there was some movement at sub-zero temperatures suggesting any escapees from the glasshouse would have the ability to move and survive a typical northern European winter.

Smooth seas do not make skilful sailors

African Proverb

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CHAPTER 1

Introduction

Glasshouse cultivation makes an important contribution to crop production as internal conditions provide protection from external environmental extremes of climate, and can be controlled to mirror optimum growth conditions (Wittwer and Castilla, 1995). The first evidence of the technology dates back to the Roman era, with more recognisable constructions in England and France recorded from the 17th century onwards (Dalrymple, 1973). Use of glasshouses is common practice in the intensive production of crops and ornamentals, as relatively small areas of production result in large yields (van de Vooren *et al.*, 1987; van Lenteren, 2000). In 2002 glasshouses covered 1,100,000 ha of the world surface, and in some countries the produce resulting from commercial glasshouses can account for up to 90% of fresh food available in winter (Tong *et al*., 2009; Boulard *et al.*, 2011).

In providing optimum conditions for plant growth, glasshouse horticulture also provides favourable conditions for pest population growth. Continuous heating promotes the expansion of invertebrate glasshouse populations regardless of time of year, and growth is usually faster than in the field (van Lenteren and Woets, 1988; van Lenteren, 2000). Dependence on insecticides increased throughout the 19th and 20th centuries, but misuse over time led to many damaging effects to human and ecosystem health, as well as the proliferation of resistance in pest populations (Isman, 2006). Safety concerns and changes to policy in Europe has resulted in the withdrawal of over half of the chemicals originally available to commercial growers

(Birch *et al*., 2011). The European Commission is promoting the use of integrated pest management (IPM) from 2014, which, through the increased use of cultural and biological control methods, aims to reduce reliance on chemical insecticides (NAS, 1969; EC, 2010; Hillocks, 2012).

Biological control is a key component of IPM, and is defined as the suppression of one species using another (Bale *et al.*, 2008b; van Lenteren, 2012). Natural enemies are usually non-native species with a narrow host range, and either predate or parasitise the pest (van Lenteren, 1997). Glasshouses are ideal environments for the use of biological control agents, as temperature and humidity can be controlled to allow optimum population growth (Paulitz and Bélanger, 2001). Use of biological control remains contentious, and candidate agents are thoroughly examined for efficacy and risks posed to the natural environment prior to release (Bale *et al*., 2008a).

In order to assess the suitability of two predatory mites, *Phytoseiulus macropilis* Banks (Acari: Phytoseiidae) and *Balaustium hernandezi* von Heyden (Acari: Erythraeidae), as candidate glasshouse biological control agents of the two-spotted spider mite, *Tetranychus urticae* Koch (Acari: Tetranychidae), for use in northern Europe, survival of a typical winter should be investigated. If the mites do not have the cold hardiness necessary to survive and reproduce, there is a lower probability of local establishment by any escapees from the glasshouse environment.

1.1. Pest Control

1.1.1. Chemical Control

Several damaging biotic and abiotic extraneous effects of chemical pesticides on the environment have been identified: damage to non-target populations, air and groundwater pollution, eutrophication within waterways and greenhouse gas emissions (Devine and Furlong, 2007). Anthropogenic manipulation of insect populations through the application of pesticides has extensive implications for ecosystems, including increased competition for diminishing resources. Distribution ranges of 24 UK farmland bird species declined between 1970 and 1990, a contraction significantly larger than other bird species associated with other habitat types (Fuller *et al.*, 1995). The decline may be attributed to the intensification of agriculture and associated costs of chemical applications in various cropping systems. Chemicals used to control arthropod pests are detrimental to a large proportion of species in the food web of agricultural ecosystems.

The application of carbamate, pyrethroid and organophosphate pesticides on non-target invertebrates, primarily those utilised as food resources for farmland birds and newly hatched chicks, has also been studied. Moreby *et al*. (2001) found that numbers of non-target Diptera, Heteroptera, Coccinellidae and Hymenoptera declined significantly between pre-spray and post-spray samples. The application of the aphicide dimethoate was found to cause a significant decrease in a population of sawflies (Symphyta: Hymenoptera), an essential food source for the grey partridge *Perdix perdix* L. (Aves: Phasianidae) (Aebischer, 1990). The results of these studies demonstrate the damage that can be caused to non-target species by the application of chemical pesticides.

Whilst non-target species suffer from the application of pesticides, there is a growing trend of increased resistance to pesticides by some target organisms. Resistance to pesticides within the United States of America currently costs the country approximately $1.5 billion annually (Pimentel, 2009). Increased resistance occurs with more frequent applications of pesticides due to the strong selection pressure for resistant genotypes in a population with a quick turnover (Georghiou, 1986).

Tetranychus urticae, known as the two-spotted spider mite or the glasshouse red spider mite (Fig. 1.1), is a significant phytophagous pest species of many commercially grown flowers, vegetables, fruit and nut trees (Driestadt *et al*., 2004). Where the species inhabits perennial ornamental plants there is a marked resistance to numerous acaricides due to recurrent application, whereas those found on edible crops, which were sprayed less due to consumer health concerns, had a much lower incidence of resistance (Stavrinides and Hadjistylli, 2009). The application of numerous insecticides to single plots can lead to a reduction in natural enemies and competing phytophagous pest populations, causing outbreaks of *T. urticae* (Dutcher, 2007). *Tetranychus urticae* is now reported to be resistant to at least 91 chemical pesticides (Whalon *et al.*, 2010).

Figure 1.1. Adult female *T. urticae* with eggs on French bean surface.

Resistance to pesticides is likely to develop through three main mechanisms: reduced sensitivity of pesticide target sites, insect detoxification and avoidance behaviour (Georghiou, 1972). *Tetranychus urticae* is known to rapidly develop resistance to organophosphate and carbamate pesticides through decreased sensitivity of the target enzyme acetylcholinesterase (Stumpf *et al.*, 2001). In susceptible strains of *T. urticae*, pesticides alter the active site leading to irreversible inhibition of the enzyme, whereas the acetylcholinesterase of resistant strains has several point mutations that render the mite impervious to certain chemical controls (Fournier, 2005).

The two-spotted spider mite metabolises pesticides through the use of mixed-function oxidase (MFO) enzymes (Brattsten *et al.*, 1977). Plants release allelochemicals in response to herbivory by *T. urticae*, which activates the detoxification enzymes to protect the mite and allow feeding to continue (Brattsten *et al.*, 1977). After treating susceptible strains of *T. urticae* and a natural predator, *Amblyseius fallacis* Garman (Acari: Phytoseiidae) with organophosphates, Mullin *et al.* (1982) found elevated MFO in the herbivores compared to the predator. These findings support the hypothesis that phytophagous mites adapt to detoxify allelochemicals emitted from plants, and can metabolise pesticides in the same fashion.

Behavioural reactions complement the point mutation and metabolic response of *T. urticae* to the application of pesticides. Mites evacuate leaves sprayed with fenvalerate or permethrin to untreated areas, whereas leaves treated with water controls do not provoke a dispersal response (Iftner and Hall, 1983). Margolies and Kennedy (1988) attributed dispersal behaviour to the anti-feedant and dehydrating effect of fenvalerate. The combination of

genetic, metabolic and behavioural responses to pesticides demonstrates the robust nature of *T. urticae* populations and the low likelihood of eradication solely through chemical control.

1.1.2. Integrated pest management

The combination of understanding of increased resistance to pesticides, widespread agro-environmental consequences and damage to human health has resulted in greater consumer demand for organic produce, defined as agriculture without chemical input (Devine and Furlong, 2007). This in turn has encouraged investment in a more environmentally friendly, conservation-oriented approach to pest reduction (Cory and Myers, 2000; Kogan and Lattin, 1993), such as use of IPM. The National Academy of Sciences (NAS) promote IPM as an ecological approach to pest management and reduction, whereby a selection of compatible techniques such as biological, cultural and chemical controls are employed to prevent pest populations exceeding the economic injury level (NAS, 1969; Eilenberg *et al.*, 2001). The economic injury level is defined as the lowest pest population density to cause economically significant damage to the crop (Buntin, 1996).

Phytoseiulus persimilis Athias-Henriot (Acari: Phytoseiidae) is a commercially available biological control agent of *T. urticae*, and is often used as a component of IPM. Trumble and Morse (1993) demonstrated that the most effective reduction of *T. urticae* on a strawberry crop was through use of both *P. persimilis* and the acaricide abamectin when compared with either treatment individually, although two-spotted spider mite has since been shown to have developed resistance against abamectin (Stumpf and Nauen, 2002). As development of resistance against pesticides is a widespread problem, the use of natural enemies as biological control agents is becoming more prevalent (van Lenteren, 2012). *Phytoseiulus persimilis* can

be combined with other biological control agents, such as *Neoseiulus californicus* McGregor (Acari: Phytoseiidae) to provide effective control of two-spotted spider mite infestations (Rhodes *et al.*, 2006).

1.1.3. Biological Control

The term 'biological control' was first introduced in to the literature in 1919 as a method of pest control utilising the natural enemies of the species (Waage and Greathead, 1988; Debach and Rosen, 1991); however, biological control has been used from as early as 304AD (Needham, 1986). Biological control is a fundamental component of IPM, and is subject to thorough investigation in order to prevent the introduction of a species that has the potential to become invasive. Biological control can be split into three broad classifications: classical, augmentative and conservation.

Classical biological control is the deliberate release of a non-native organism in the environment with the aim of permanent establishment in order to control a pest population. Use of this practice was first recorded in 304AD, whereby the ant *Oecophylla smaragdina* Fabricius (Hymenoptera: Formicidae) was released into orange trees in China to control citrus-feeding invertebrates (Needham, 1986). Biological control agents have usually co-evolved with the pest species, and thus classical biological control has a long term, self-sustaining nature.

One of the more prolific cases of classical biological control of an invertebrate occurred in 1868, in Californian citrus groves. The cottony-cushion scale, *Icerya purchasi* Maskell (Homoptera: Margarodidae), had been accidentally introduced to Menlo Park, Northern

California, on *Acacia* trees imported from Australia, and posed an enormous threat to the Californian citrus industry (DeBach and Rosen, 1991). Research in the native environment of *I. purchasi* revealed two candidate biological control agents: a parasitic fly, *Cryptochaetum iceryae* Williston (Diptera: Cryptochaetidae); and the vedalia beetle, *Rodolia cardinalis* Mulsant (Coleoptera: Coccinellidae). Control by *C. iceryae* is prevalent in cooler regions, where success of *R. cardinalis* is limited by lower temperatures (Caltagirone and Doutt, 1989). *Rodalia cardinalis* eggs will not hatch at 10°C, and 100% development of egg to adult is limited unless temperatures meet the threshold of 22°C (Grafton-Cardwell *et al.*, 2005). However, the control of *I. purchasi* in California is frequently solely attributed to *R. cardinalis* as the beetles visibly swarmed over citrus trees, and within six months the cottony-cushion scale had been practically eradicated at a cost of $1500 (Thórarinsson, 1990; DeBach and Rosen, 1991). Persistence of *R. cardinalis* in Californian citrus groves has been reported since its initial introduction, and *I. purchasi* is consequently maintained at a low population density (Murdoch *et al.*, 2006).

Classical biological control depends on establishment of the agent in an exotic ecosystem, and therefore inherently poses a risk to the environment. Each candidate organism should be thoroughly investigated prior to release in order to identify and negate any negative impacts on the native environment. For example, two invasive species of prickly pear, *Opuntia* spp., occupied 60,000,000 acres of Australia, and were reduced to less than 10% of the former population by *Cactoblastis cactorum* Berg (Lepidoptera: Pyralidae) after a series of host specificity tests were undertaken to ensure native vegetation populations were unaffected (Dodd, 1936). The success of this biological control agent was widely celebrated, and the moth was imported to many other regions to reduce non-native *Opuntia* outcrops, such as the

Caribbean. Migration or accidental carriage through trade with the United States of America led to the discovery of a population in the Florida Keys (Johnson and Stiling, 1998). Florida has six native species of prickly pear, of which *Opuntia corallicola* Small (Magnoliopsida: Cactaceae) is extremely rare, and was thought to be restricted to 12 individuals on one Key at the time of *C. cactorum* colonisation (Stiling *et al.*, 2004). *Opuntia corallicola* was preferentially targeted by *C. cactorum*, and to date the only effective response has been to plant the species on six isolated Keys (Stiling and Moon, 2001). This case study demonstrates the risk of a hasty introduction of a non-native invertebrate to an environment where establishment is possible, without prior investigation of non-target effects.

Whereas classical biological control agents are required to successfully establish and reproduce in the affected area, augmentative biological control is the application of an exotic natural enemy in areas where abiotic factors prevent prolonged survival and reproduction (van Lenteren and Bueno, 2003). Growers of glasshouse vegetables make more use of biological control compared with any other users of the technology, and proliferation of augmentative biological control has lead to a decrease in the use of chemical pesticides in these environments (Parrella, 2008). Eilenberg *et al.* (2001) distinguish between inoculative and inundative forms of augmentative biological control, whereby inoculation agents are released to control a pest with reproduction of the agent over several generations but without permanent establishment; and inundation agents achieve control without production of a following generation *in situ*.

The first recorded use of augmentative biological control was prior to 1917 (van Lenteren, 2006). The southern Californian citrus industry was threatened by the mealybug

Pseudococcus calceolariae Maskell (Homoptera: Pseudococcidae) and applications of chemical pesticides proved ineffective. The population was eventually brought under control by a known natural enemy, *Cryptolaemus montrouzieri* Mulsant (Coleoptera: Coccinellidae), which was unable to survive the local winter temperatures and thus was mass-reared in insectaries and intermittently released as a biological control agent (Luck and Forster, 2003). Low temperatures prevented permanent establishment of the species, and therefore counteracted the extent to which any native non-target species may have been affected.

Conservation biological control comprises of the alteration of the environment in which natural enemies operate in order to enhance their survival, reproduction and predatory performance (Barbosa, 1998). This may include the provision of shelter and alternative food sources in field margins with an assortment of wild flower species which enhance native natural enemy populations (Baggen *et al.*, 1999; Kean *et al.*, 2003; Fielder and Landis, 2007). Increasing size and age of field margins has been correlated with an increased population size of predatory arthropods (Denys and Tscharntke, 2002). Populations of phytoseiid predators have increased in citrus orchards where conservation management strategies such as pollen supplementation and sown non-crop covers are provided with the aim of reducing *T. urticae* infestations; however, the supply of pollen tends to attract generalist species, resulting in fewer spider mite specialists (Grafton-Cardwell *et al*., 1999; Aguilar-Fenollosa *et al.*, 2011). This type of control is less specifically targeted than classical or augmentative, but achieves pest control at a landscape scale, usually without any pesticide input (Tscharntke *et al.*, 2007).

1.2. Advantages and disadvantages of biological control

Several reasons have been proposed for grower interest in biological control as opposed to use of pesticides: there are no phytotoxic repercussions for the crop; the release of a biological control agent is more efficient; and there is no obligatory lag in time between application of the agent and harvest, whereas health and safety regulations require a delay between spraying and collection of the crop (van Lenteren, 2000). The development of chemical pesticides costs significantly more than biological control, $180 million compared with $2 million respectively, and is less target-specific (Bale *et al.*, 2008b). In more than 50% of instances where multiple biological control agents have been introduced, a single species has been responsible for the reduction of the pest population, demonstrating the system of biological control to be cost effective (Denoth *et al.*, 2002).

Despite the benefits of biological control, there are also costs to consider. It is widely believed that introduced exotic species are one of the five most destructive forces affecting ecosystems, along with overexploitation, pollution, habitat destruction and disease (Wilcove *et al.*, 1998). The introduction of a non-native species could have a significant impact on the local community as Salo *et al*. (2007) found that an exotic predator has double the impact on a native prey species compared with an indigenous predator.

Collier and Van Steenwyk (2004) reviewed the ecological limitations of augmentative biological control, and found factors such as enemy dispersal, refuge for the pest and intraguild predation will affect the success of the agent. Efficacy of the candidate biological control agent should be investigated and combined with a risk assessment in order to

determine suitability prior to release. The risk assessment should comprise of several factors, including an examination of survival of the candidate biological control agent outside of the glasshouse. It is important to determine whether any escaping individuals would survive the local climate as the combination of favourable climate and prevalence of the enemy escape hypothesis, where co-evolved predators will be absent from the environment, leave few limiting factors on population growth rate (Colautti, 2005). *Neoseiulus californicus* is native to North America, and was released into glasshouses in the UK in 1991 as a biological control agent of *T. urticae* (Hart *et al*., 2002b). Wild populations have since been recorded on strawberry and in orchards, and survival of winter temperatures attributed to the ability to enter diapause (Jolly, 2000). Rigorous investigation into the behavioural and physiological characteristics of a candidate biological control agent is therefore necessary prior to release in order to prevent undesirable permanent establishment.

1.3. Tritrophic Interactions in Biological Control

Whilst under attack from herbivores, plants have two defensive options: direct production of toxins and anti-feedants, and indirect defence through the attraction of predators. Herbivore-induced plant volatiles (HIPVs) are an indirect defence against colonisation of phytophagous species, whereby different infochemicals are released by the plant to indicate the presence of adults or eggs to natural predators (Fatouros *et al.*, 2008; Dicke and Baldwin, 2010). Janssen (1999) used an olfactometer to demonstrate the marked preference of *P. persimilis* for the volatiles emitted from *T. urticae* infested plants than clean plants. Alborn *et al.* (1997) found that isolated herbivore oral secretions applied to damaged leaves induced the release of the same HIPVs as actual insect feeding, and attracted natural predators. Mechanical damage without the addition of herbivore oral secretions prompts the release of different plant

volatiles, and so it can be inferred the oral secretions trigger HIPV emission (Dicke and van Loon, 2000).

In addition to advertising the presence of herbivores to predators, emission of HIPVs has beneficial effects on surrounding plants. Arimura *et al.* (2000) demonstrated that release of HIPVs from *T. urticae* infested lima bean plants, *Phaseoulus lunatus* L. (Fabaceae), activated five defence genes in leaves of neighbouring conspecifics, rendering them more resistant to attack. Tritrophic systems are mutually beneficial for both plant and predator, and are often exploited by commercial agricultural growers using biological control.

Choh *et al*. (2006) found that HIPVs emitted from *T. urticae* infested *P. lunatus* caused increased production of extrafloral nectar by intact conspecific plants. Extrafloral nectar is a source of sugars, amino acids, lipids and a number of micronutrients, and as such is attractive to predatory mites with generalist diets as a short term energy supply (Lundgren, 2009). Plants may also provide protective structures such as acarodomatia, in the form of either small cavities or dense extrusions of non-glandular trichomes on the leaf surface. The presence of non-glandular trichomes can be beneficial for small invertebrate biological control agents, such as mites, as these structures offer protection from larger invertebrate predators and against intraguild predation of eggs (Norton *et al.*, 2001; Roda *et al.*, 2000). As well as shelter from other predators, the presence of trichomes will offer a more humid environment, protecting eggs and individuals from desiccation (Krips *et al.*, 1999). Experimental removal of trichomes from the abaxial vein axils of *Viburnum tinus* L. (Caprifoliaceae) significantly reduced the size of a population of beneficial mites (Walter and O'Dowd, 1992).

Despite the advantages of a tritrophic system for phytoseiid mites, there are also costs. Tomato, *Solanum lycopersicum* Miller (Solanaceae), is a common target of *T. urticae* and as such is subject to biological control regimes using predatory mites. In contrast to non-glandular trichomes which offer protection to beneficial mites, tomato leaves often have a dense covering of glandular trichomes which produce a sticky exudate that are deleterious to the most widely used predators of *T. urticae*: *P. persimilis* and *N. californicus* (Walter, 1996; Cédola *et al.*, 2001). High trichome densities on other commercially grown plants such as *Gerbera jamesonii* L. (Asteraceae) reduce the predation rate of *P. persimilis* compared to low trichome cultivars (Krips *et al.*, 1999). It is therefore important to account for any deleterious effects of the host plant on a candidate biological control agent, and ascertain whether the agent is effective on the plant surface prior to release.

1.4. Selection and legislation

1.4.1. Identifying candidate biological control agents

Bioprospecting, the investigation into the potential economic value of biodiversity, is contributing to the growth in numbers of species used as biological control agents (Beattie *et al.*, 2005). Once a candidate agent has been selected from its native environment, it is essential to determine whether it will have any deleterious effects on an exotic ecosystem. The centrifugal phylogenetic method assesses the likelihood of a candidate biocontrol agent to predate a non-target host (Wapshere, 1974). The agent is exposed to different organisms starting at the closest relations to the target, eventually moving to more distantly related species, and any non-target attack is assessed (Messing and Wright, 2006).

Ideal agents are monophagous, but oligophagous agents may be utilised where affected non-target species do not occur in the release area (Kelch and McClay, 2003). Some studies have highlighted the disparity between fundamental host range investigated in the laboratory, and ecological host range of a natural enemy. Haye *et al.* (2005) found that the parasitoid *Peristenus digoneutis* Loan (Hynmenoptera: Braconidae) parasitized all available non-target hosts in the laboratory, but less than 1% of those species in the field. Similarly, several potential tachinid and braconid biological control agents of *Diabrotica virgifera virgifera* LeConte (Coleoptera: Chrysomelidae) have wide fundamental host ranges, but very narrow ecological host ranges (Toepfer *et al.*, 2008). These studies emphasize the importance of investigation in the field, as well as the laboratory, in order to define the ecologically relevant host range of a candidate biological control agent.

1.4.2. Pre-release risk assessment

There are currently no Europe-wide legislative restrictions governing the use of invertebrate biological control agents. As developmental costs are low, and there are no strict guidelines to follow, twenty commercial suppliers of biological control agents are active in Europe (van Lenteren, 2012). There are several organisations and regulations to contain and monitor the use of biological control agents; however, there are no harmonised guidelines for EU countries. Through the 'Evaluating Environmental Risks of Biological Control Introductions into Europe' (ERBIC) project, it was found that only 1.5% of classical biological control introductions had research conducted into the non-target effects of the agent (Lynch *et al.*, 2001). As invertebrate migration will occur irrespective of country boundaries or respective legislation, a harmonised scheme of regulation is essential to prevent the spread of invasive species (Bale, 2011).

The regulatory processes in Australia, New Zealand, Canada and the USA represent a basis for more synchronised legislation; each country requires investigation into the environmental impact of the species, and a final science-based decision is made regarding application approval (Hunt *et al*., 2008). The European and Mediterranean Plant Protection Organisation (EPPO) and Organisation for Economic Cooperation and Development (OECD) collaborated to clarify the information necessary to assess suitability of a candidate agent prior to release. This includes: biology and ecology of the agent; effects on human health; environmental risks, specifically focussing on direct and indirect effects; and potential dispersal leading to establishment (OECD, 2004). Using a framework based on the these parameters, it is possible to calculate the risk index of a particular candidate biological control agent, thus allowing the processes and decisions regarding invertebrate biological control agent suitability for release to be harmonised, and based upon empirical data (van Lenteren *et al.*, 2003).

In 2004, the European Commission proposed all previous regulation guidelines to be collated. The International Organisation for Biological Control/West Palearctic Regional Section (IOBC/WPRS) set up the 'Commission on Harmonised Regulation of Invertebrate Biological Control Agents' (CHIBCA) to link all previous work on regulation of biological control, and to produce one document containing all relevant information and guidelines (Bigler *et al*., 2005a). It was expected that these guidelines would standardise regulations across the EU within a couple of years (Bigler *et al*., 2005b).

The Regulation of Biological Control Agents project (REBECA) was subsequently formed to investigate the management and supervision of biological control agents within the EU. Through this project, the effectiveness of current risk assessment practices employed within separate countries of the EU was evaluated, and recommendations concerning the release of invertebrate biological control agents, including the standardization of release parameters across the EU were discussed (Loomans, 2007). Despite the recommendations of the IOBC/WPRS CHIBCA, only eight of the nineteen countries investigated had standard regulatory processes in place, including the UK; five countries had no regulatory system in place at all (Bigler *et al.*, 2005b). Failure to recognise the importance of a harmonised system has already resulted in the spread of an invasive species, *Harmonia axyridis* Pallas (Coleoptera: Coccinellidae), to seven European countries with no history of deliberate release (Brown *et al*., 2008).

Without harmonisation of regulation, species introductions will continue in unregulated countries without investigation of the environmental impact, and therefore pose a risk of becoming invasive and migrating to localities of increasing distance from sites of release. Environmental Risk Assessments (ERAs) are essential, as the ability of a candidate biological control agent to establish will determine the degree to which non-target species are affected (van Lenteren *et al.*, 2003). Regulations in the UK are based on sections 14 and 16 of the 'Countryside and Wildlife Act 1981', which prohibit release of non-native species without a license. An 'application for license to release a non-native species for biological control purposes in England' assesses the risks presented by a species based upon the guidelines set by the REBECA project, and is reviewed by the Food and Environment Research Agency (FERA).

The licensing application follows an ordered process, requiring examination of the establishment potential, host range and dispersal ability of the arthropod. Any candidate biological control agent that does not meet the safety requirements of a particular level of the hierarchy is likely to be rejected (Fig. 1.2) (van Lenteren *et al*., 2006). Establishment potential is the first factor to be assessed. If establishment of a glasshouse biological control agent is prevented by abiotic factors in the country of release only transiency of direct and indirect effects through the summer period need be assessed to determine safety for release (van Lenteren *et al*., 2006; Bale, 2011).

1.4.3. Access and benefit sharing

The Convention on Biological Diversity (CBD) is an international treaty consisting of three primary objectives: conservation of diversity, sustainable use of biodiversity, and equitable sharing of the benefits arising from use of genetic resources (CBD, 1993). The European Union has ratified the CBD, which not only obliges signatories to prevent and control introductions of non-native species, but also provides sovereign rights over genetic resources within the country (Hunt *et al*., 2008; Cock *et al*., 2010). The intention was to limit the freedom with which foreign commercial organisations could exploit a genetic resource within a biodiversity-rich country (Jinnah and Jungcurt, 2009).

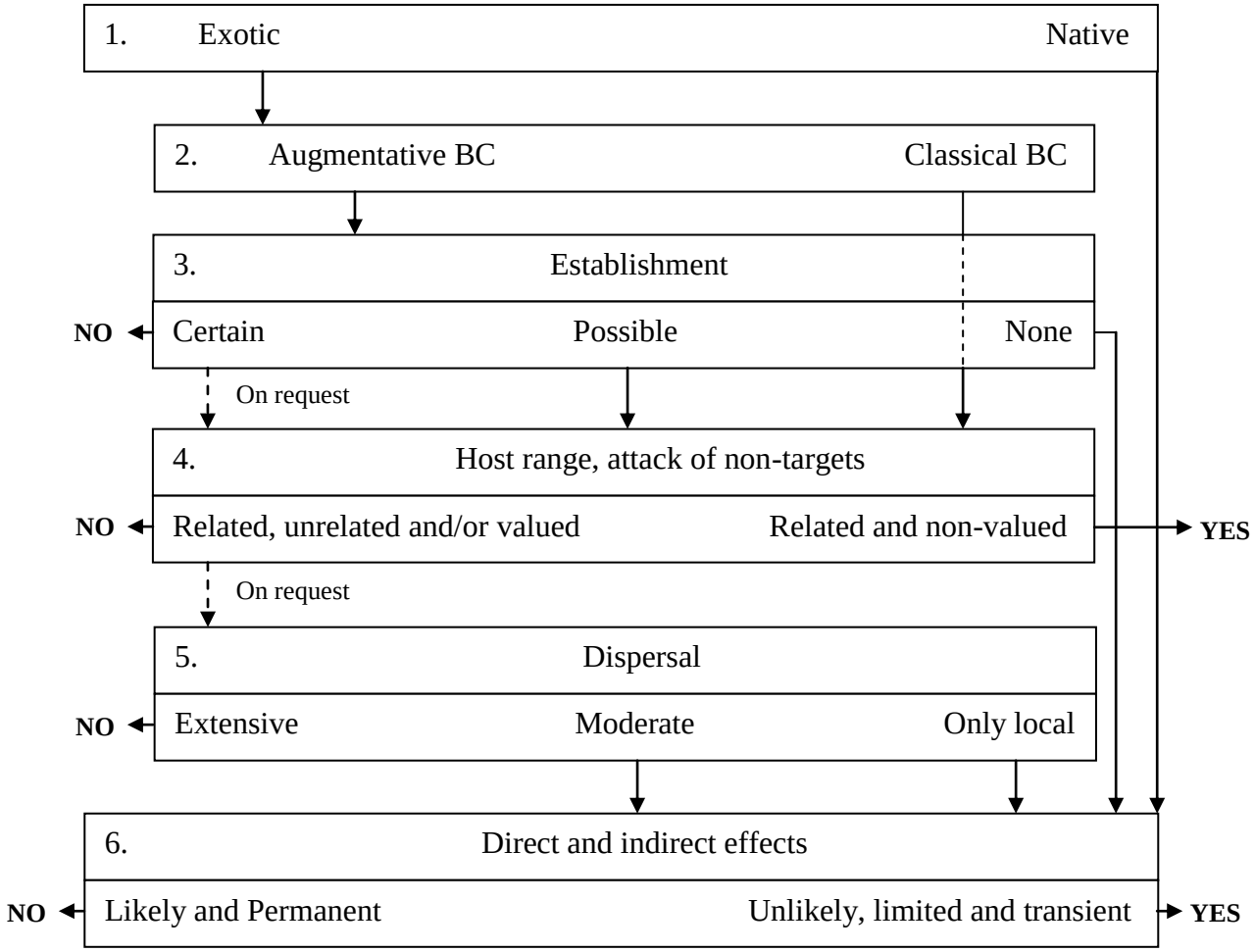

Figure 1.2. Environmental risk assessment for arthropod biological control agents, where NO: release is not recommended; YES: release is recommended (from van Lenteren *et al*., 2006).

Bioprospecting by companies, such as commercial producers of biological control, is now overzealously regarded as "biopiracy", and as a result, access to biological resources can only be gained through the prior informed consent of the host nation (ten Kate, 2002; Siebenhüner and Suplie, 2005; Barratt, 2009). There is growing concern that the restriction of access will damage advancement within biological control; for example, efforts to find a natural parasitoid of *Liriomyza huidobrensis* Branchard (Diptera: Agromyzidae) have been halted due to the restrictions on sharing specimens between Europe and South America (Cock *et al*., 2010).

1.4.4. Post-release monitoring

Post-release monitoring is the responsibility of the importer, and may be examined by the National Plant Protection Organisation (FAO, 2006). EPPO maintains a list of biological control agents applied within Europe that have since exhibited non-target dispersal and attacked non-target hosts. In January 2014 the list comprised *Cales noacki* Howard (Hymenoptera: Aphelinidae), *Lysiphlebus testaceipes* Cresson (Hymenoptera: Aphididae) and *H. axyridis* (EPPO, 2014).

It is important to supervise the exotic organism in order to gauge whether the desired control is occurring and if there have been any deleterious effects on the ecosystem. The more rigorous the post-release monitoring, the more efficiently any problems can be identified. Two experimental methods of post-release monitoring have been proposed: non-target species are sampled in areas where the biological control agent has been released; or the 'enrichment' method, whereby non-target individuals are placed in an environment where the biological control agent is known to occur, and the area is subsequently sampled (Babendreier *et al.*, 2005).

The requirements for licensing glasshouse biological control agents in northern European countries such as the UK should negate the necessity of post-release monitoring. Pre-release assessments that examine the cold hardiness will prevent the introduction of a species capable of winter survival and potential establishment.

1.5. Physiology of cold hardiness

The body temperature of poikilothermic organisms, such as arthropods, fluctuate directly with temperature, rendering them susceptible to changes in local climate (Powell and Logan, 2005). The study of cold hardiness of a candidate biological control agent is therefore a useful indicator of whether the insect could establish, as climate will act as a density-independent limiting factor on population growth (Bale, 1991). Cold hardiness is defined as the combined characteristics required to avoid the damaging effects of low temperature potentially experienced during winter (Bale, 1987). A species may also have the ability to 'recognise' the cues of oncoming winter conditions and enter a hypometabolic state, termed diapause, to increase the likelihood of survival. To prevent establishment outside of the glasshouse, the physiology of candidate biological control agents should be examined for the potential to survive typical winter conditions. If the species encounter temperatures above the developmental threshold during winter, the life cycle can be completed, a surviving generation can be produced and long-term establishment is possible (Hatherly *et al.*, 2005a).

In order to survive decreases in temperature which could lead to mortality, two principal strategies of insect cold hardiness have been extensively discussed: freeze tolerance and freeze avoidance (Salt, 1961; Baust, 1981; Bale, 1987; Lee, 1989). The central elements of each strategy of cold hardiness are shown in Fig. 1.3. The two strategies of survival will enable an insect to develop from egg to adult during extended cold periods such as winter. There is some debate as to whether a simple division between freeze tolerant and freeze avoiding strategies is imprecise, and a more broad scale of cold hardiness operates.

Five gradations of insect cold hardiness have been proposed: freeze tolerant, freeze avoiding, chill tolerant, chill susceptible and opportunistic survival (Bale, 1993). Each of these strategies, and the cues for diapause induction are discussed later in further detail.

1.5.1. The supercooling point

Both freeze avoidance and freeze tolerance rely on the process of supercooling, whereby small volumes of bodily fluid remain aqueous despite being cooled below the melting point, until the liquid freezes spontaneously (Lee, 1989). The moment of crystallisation, or 'supercooling point' (SCP), can be measured by the release of latent heat as the whole body freezes (Fig. 1.4). Whereas freeze avoiding insects will not survive beyond instant freezing, freeze tolerant species are defined as those that continue to develop successfully following a period of time at temperatures below the SCP once the temperature has increased (Bale, 1991).

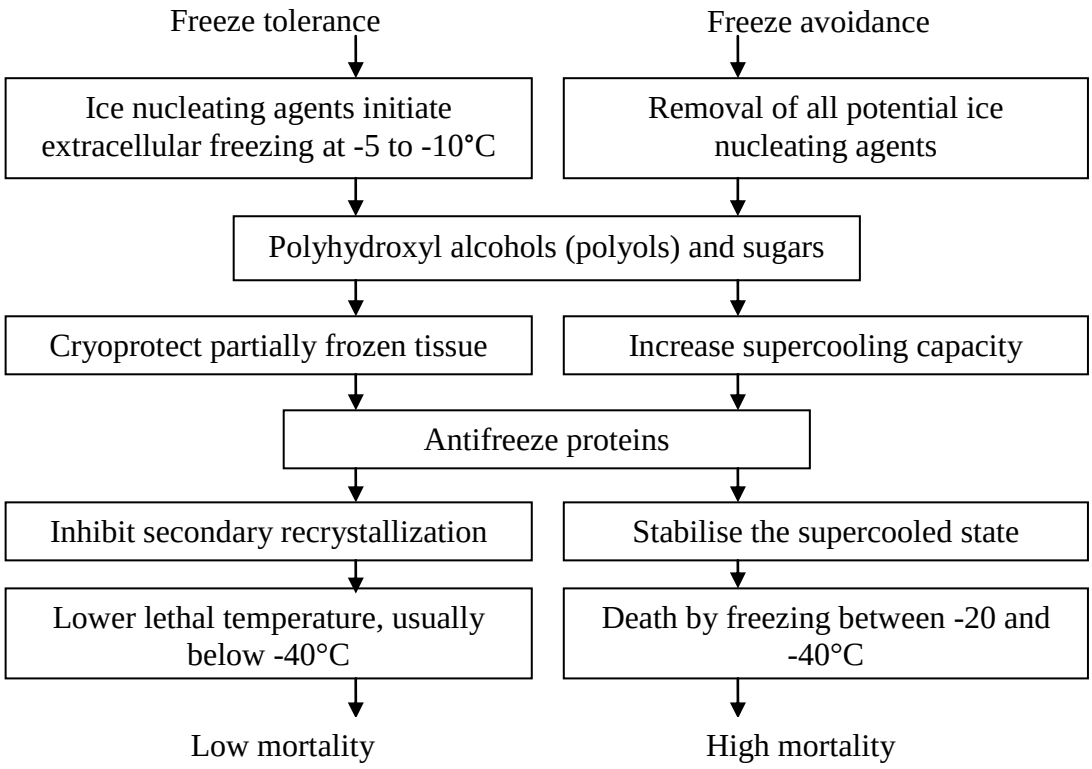

Figure 1.3. Systems of freeze tolerance and freeze avoidance in insects (from Bale, 2002).

1.5.2. Freeze tolerance

Freeze tolerance arises through the action of ice nucleators that accumulate in the body prior to extreme cold (Salt, 1961). Arthropods utilising this system have been reported to freeze at higher sub-zero temperatures regardless of the bodily concentration of polyhydric alcohols such as glycerol and sorbitol, which act to lower the supercooling point in freeze avoiding insects (Miller and Smith, 1975; Zachariassen and Hammel, 1976). Freeze tolerant insects instigate and direct ice formation in extracellular fluid spaces within the body through the use of haemolymph protein ice nucleators (Duman, 2001; Storey, 1990); and also utilise antifreeze proteins as cryoprotectants in order to prevent damage caused by recrystallisation during the warming period following winter.

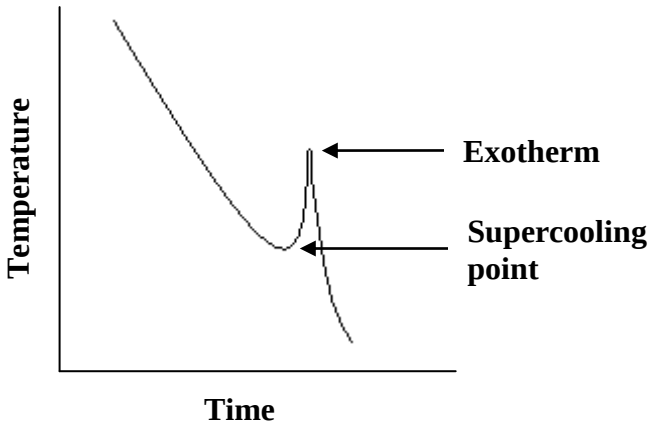

Figure 1.4. Insect body temperature response to decreasing external temperature, where the supercooling point indicates release of latent heat as the whole insect freezes (Lee, 1989).

A freeze tolerant strategy ensures that the supercooling point remains only a few degrees below zero (Zachariassen, 1985; Bale and Hayward, 2010). *Syrphus ribesii* L. (Diptera: Syrphidae) is a strongly freeze tolerant species, as the mean supercooling point is -6.9 ± 0.6°C, with more than 70% larvae still alive at -35°C (Hart and Bale, 1997). There is a large

discrepancy between the relatively high sub-zero supercooling point and the lower lethal temperature, defined as the lowest temperature to kill 50% of a population (Cloudsley-Thompson, 1962), ensuring survival of the freeze tolerant species in lower sub-zero conditions.

1.5.3. Freeze avoidance

Supercooling is the foremost defence of the freeze avoidance strategy. The process of supercooling is aided by the removal of protein ice nucleators and any other ice nucleating agents within the body. Debate appears to arise over what constitutes an ice nucleating agent, as Baust and Rojas (1965) argue that a lack of comprehensive evidence undermines the perceived relationship between gut content and supercooling, whereas Salt (1968) maintains that contents of the gut will act as ice nucleating agents, and insects can manipulate this in order to increase or decrease their supercooling points. Sømme (1982) proposes that ice nucleation may not arise from the gut content, but from the digestive juices that are secreted when food is consumed by the insect.

The supercooling point of a freeze avoiding insect usually reflects the lower lethal temperature, and insects will therefore act to reduce potential ice nucleating agents, resulting in depression of the supercooling point and prolonged survival. Thermal hysteresis antifreeze proteins are often employed to lower the supercooling point of the haemolymph through inhibition of ice nucleating agents (Block and Duman, 1989). Accumulation of polyols, such as glycerol, within the body can allow the supercooling point to be depressed to as low as -40°C (Zachariassen, 1985; Storey, 1990).

Furthermore, Duman (2001) postulated that the waxy cuticle of the insect acts as an initial defence against inoculative freezing from external ice, which may otherwise trigger internal ice nucleation and increase mortality. Larsen and Lee (1994) found that rain or dew, wetting the body of *Danaus plexippus* L. (Lepidoptera: Danainae) significantly raised the supercooling point, resulting in lower cold hardiness of the individual.

It has been argued that the strategy of freeze avoidance does not account for insect mortality at sub-zero temperatures prior to reaching the supercooling point, and thus the classifications of chill tolerance and susceptibility have been proposed to explain pre-freeze mortality (Bale, 1993).

1.5.4. Chill tolerance

Chill tolerant species show some pre-freeze mortality, yet can survive long periods at low temperatures (Bale, 1993). *Neoseiulus californicus* is classified as a chill tolerant species as the species survived several months at 5°C in laboratory conditions, yet 90% of an experimental sample died at temperatures above the mean supercooling point of -21.6°C (Hatherly *et al.*, 2005a; Hart *et al.*, 2002b). The laboratory findings were supported by field trials, as an unfed treatment of the non-diapausing *N. californicus* strain survived three months in UK winter exposures (Hart *et al.*, 2002b).

1.5.5. Chill susceptibility

Chill susceptible species can survive at temperatures below the developmental threshold, but will have a high mortality rate at relatively high sub-zero temperatures (Bale, 1993). *Drosophila melanogaster* Meigen (Diptera: Drosophilidae) has been classified as chill

susceptible as the species has been found to die between 0 and -5°C, considerably higher temperatures than the supercooling point of -20°C (Czajka and Lee, 1990; Colinet *et al.*, 2010). Aphids are also considered to be chill susceptible species, as Bale (1996) found the lower lethal temperatures of *Myzus persicae* Sulzer, *Sitobion avenae* Fabricius and *Rhopalosiphum padi* L. (Hempitera: Aphididae) to be approximately 15°C above the supercooling points of -24 to -27°C. Chill susceptible species, such as aphids, may be able to survive adverse conditions in the short term if the process of acclimation is employed (Powell and Bale, 2004).

1.5.6. Opportunistic survival

Opportunistic survival differs to chill susceptible species as insects employing this strategy have a high mortality in exposures to temperatures below the developmental threshold (Bale, 1993). Mortality of *Musca domestica* L. (Diptera: Muscidae) was high during exposures to 0°C, reaching 12% after 2h, and 90% after a 4 day exposure (Coulson and Bale, 1990). Sheltered structures can provide microclimates in which these species can retreat from adverse environmental conditions, and some predatory mites such as *N. californicus*, *Neoseiulus makawa* Ehara and *Neoseiulus womersleyi* Schicha (Acari: Phytoseiidae) are known to exploit these opportunities (Kawashima and Jung, 2010a,b).

1.6. Measurement of thermal biology

1.6.1. Acclimation

Acclimation in the laboratory, or acclimatization in the field, is a long term exposure to a sublethal temperature that contributes to prolonged survival of low or sub-zero temperatures (Fields, 1992; Hoffman *et al.*, 2003). For example, *D. melanogaster* acclimated to 15°C was

able to find and utilise food resources in field releases in Denmark during winter, whereas those reared at 25°C perished (Kristensen *et al.*, 2008). However, efficacy of acclimation may depend on the conditions following the treatment. Studies of several species of *Drosophila* demonstrated that an acclimation treatment of 32 days at 11°C had higher survival in a subsequent exposure to 2°C than the same acclimation period at 15°C, and many previous studies of acclimation have successfully used treatments exposured to temperatures between 0 to 11°C (Hoffman *et al.*, 2003).

Cold acclimation should afford an insect protection from accumulated chilling injury, where there is additional oxidative stress overwhelming the antioxidant defence system (Renault *et al.*, 2004; Rojas and Leopold, 1996). Oxidative stress in *Drosophila* is thought to elicit responses such as cellular senescence and apoptosis, and can be lethal if the stress is prolonged (Terhzaz *et al.*, 2009).

1.6.2. Lethal temperature

Lethal temperatures are typically defined as the lowest or highest temperatures to kill a predefined proportion of a population (Cloudsley-Thompson, 1962). The results of lethal temperature experiments can demonstrate the level of pre-freeze mortality in freeze avoiding species, and aid the identification of chill tolerance or susceptibility in an organism (Bale, 1993). The lethal temperatures of several predatory mites have been measured as part of an investigation into their suitability as glasshouse biological control agents (Hart *et al.*, 2002b; Hatherly *et al.*, 2004; Allen, 2010).

1.6.3. Lethal time

The lethal time ($LTime_{50}$) is defined as the time taken to kill 50% of the population at a given temperature (Hatherly *et al.*, 2005a). The experiment assesses the effect of chronic exposure to sub-lethal temperatures. A strong positive correlation has been found between $LTime_{50}$ at 5°C and survival time of a range of non-native biological control agents in the field (Hatherly *et al.*, 2005a; Bale *et al.*, 2008b) (Fig. 1.5). Candidate biological control agents pose a higher risk of establishment in the UK if both the $LTime_{50}$ and field trial survival values are high, as this indicates that the species will be more tolerant of typical northern European winter temperatures.

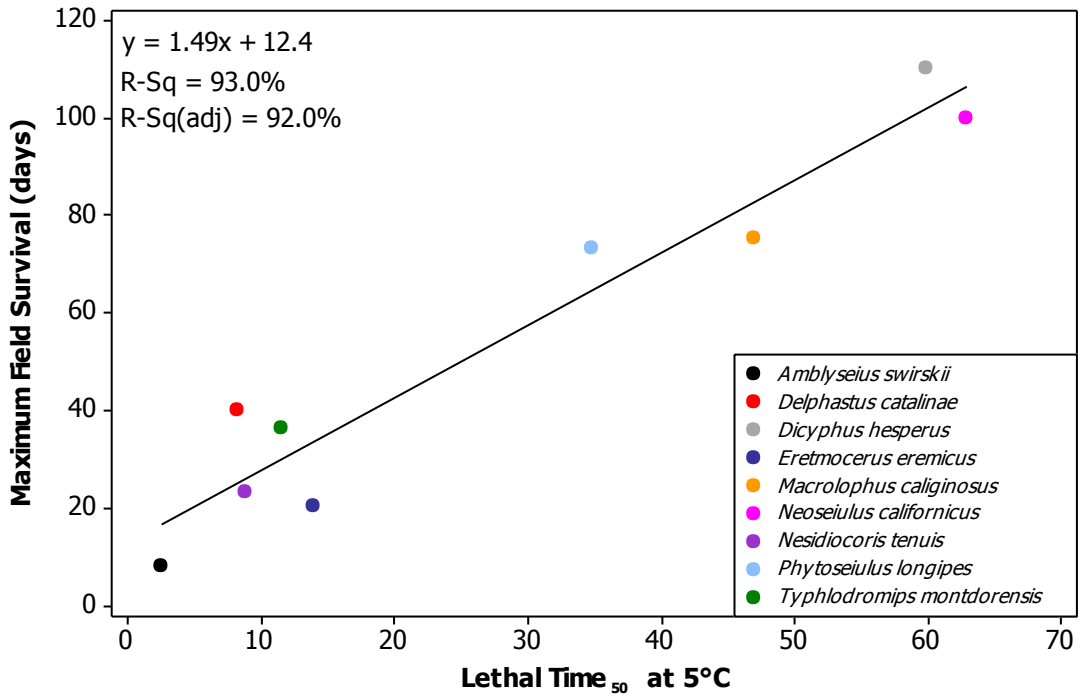

Figure 1.5. Correlation between $LTime_{50}$ at 5°C and maximum unfed field survival time of several biological control agents. Sources of data: *Amblyseius swirskii* (Allen, 2010); *Delphastus catalinae* (Tullett, 2002); *Dicyphus hesperus* (Hatherley *et al.*, 2008); *Eretmocerus eremicus* (Tullett *et al.*, 2004); *Macrolophus calignosus* (Hart *et al.*, 2002a); *Neoseiulus californicus* (Hart *et al.*, 2002b); *Nesidiocoris tenuis* (Hughes *et al.*, 2009); *Phytoseiulus longipes* (Allen, 2010); *Typhlodromips montdorensis* (Hatherley *et al.*, 2004).

Dicyphus hesperus Knight (Hempitera: Miridae) and *N. californicus* pose the most risk as $LTime_{50}$ and field trial survival values are high, substantiated by the establishment of wild populations of *N. californicus* in the UK since its release as a biological control agent (Jolly, 2000) (Fig. 1.5). The relationship between $LTime_{50}$ and maximum field survival time appears to be a powerful tool to evaluate any candidate arthropod biological control agent, and should be incorporated into data used in application for a license for release. Although laboratory measures of lethal time at 5°C provide a reliable indication of survival in the field, the experiment does not account for the variability of northern European winter temperatures between years (Luterbacher *et al*., 2004).

1.6.4. Diapause

Poikilothermic metabolisms are susceptible to adverse changes in climate, and some ectotherms are able to enter a hypometabolic state to cope with seasonal changes in temperature (Storey and Storey, 2007). In arthropods the suspension of development and redirection of resources prior to more hostile environmental circumstances in order to increase likelihood of survival is termed 'diapause' (Koštál, 2006). It is a genetically programmed response which may be expressed either at a particular life stage of every generation ('obligate' diapause), or if particular environmental conditions are encountered ('facultative' diapause) (Bale and Hayward, 2010; Denlinger, 2002).

Photoperiod and thermoperiod are principal cues for entry into diapause, as decreases in day length and temperature over time are an indication of future hostile conditions (Tauber and Tauber, 1973; Tauber and Tauber, 1976). Photoperiod is the primary cue in initiating diapause in temperate regions above 30° N latitude, and the critical day length is defined as the

photoperiod that induces diapause in 50% of the population (Bale and Hayward, 2010; McWatters and Saunders, 1997). Upon encountering diapause-inducing conditions, some species upregulate the expression of several heat shock proteins, which bind to other proteins and mitigate the deleterious effects of cold stress (Rinehart *et al*., 2007).

Some phytoseiid species have the capacity to survive winter conditions in the microclimates of crevices in tree bark, soil, leaf litter, hibernation cocoons and fruit pedicels (Veerman, 1992; Broufas *et al.*, 2002). Overwintering in these sites has been recorded in both diapause and non-diapause strains of *N. californicus* (Kawashima and Jung, 2010a,b). Phytoseiids generally enter facultative diapause, prompted by a decrease in daylight hours indicating the onset of winter (Morewood, 1993). The critical day length of some phytoseiids known to enter diapause is shown in Table 1.1.

Table 1.1 The critical day length of phytoseiid species known to enter diapause.

Species	**Critical Day Length (h)**	**Reference**
Typhlodromips montdorensis	11	(Hatherly *et al.*, 2005b)
Euseius finlandicus	12	(Broufas *et al*., 2006)
Metaseiulus occidentalis	11	(Hoy, 1975)
Typhlodromus pyri	12.5 – 13.5	(Fitzgerald and Soloman, 1991)
Neoseiulus fallacis	11.75 - 12	(Rock *et al.*, 1971)
Amblyseius cucumeris	12.45	(Morewood and Gilkeson, 1991)
Amblyseius potentillae	14.5	(van Houten and Veenendaal, 1990)

Male phytoseiid mites tend to perish at the onset of adverse climatic conditions, whereas gravid females are known to survive through diapause which is expressed as a hiatus in egg-laying (Wysoki and Swirski, 1971). However, the ability to diapause is not ubiquitous within phytoseiids, and those originating from tropical and sub tropical regions show homodynamic development (Veerman, 1992).

In some species, diapause is associated with a change in physical appearance: the body colour of *Nezara viridula* L. (Heteroptera: Pentatomidae) changes from a vivid green to a dull yellow or brown (Musolin, 2012); the dorsal shield of diapausing members of the Phytoseiidae appears more flattened, and become more pale in colour (Veerman, 1992). In addition to the measured physiological responses during diapause experiments, any phytoseiid species in diapause should be more recognisable compared to their non-diapausing conspecifics.

Diapause has been described in *Balaustium mumorum* Hermann and *Balaustium putmani* Smiley (Acari: Erythraeidae), which both express diapause at the egg stage (Putman, 1970; Belozerov, 2008a). To date, studies on this genus have been field based and therefore observations of hibernal diapause have not included information on the critical day length. *Balaustium medicagoense* Meyer and Ryke (Acari: Erythraeidae), a crop pest in Australia, was observed to have two generations a year with a likely diapause during summer, as no active individuals were observed during November to April (Arthur *et al*., 2011).

It is important to include investigation of diapause in the risk assessment of a candidate biological control agent. Increased cold hardiness is closely linked with this physiological adaptation, and some species exhibit different responses to measures of cold tolerance when in diapause (Pullin, 1996). For example, the supercooling points of diapause and non-diapause larvae of *Adoxophyes orana* Fischer von Rosslerstamm (Lepidoptera: Tortricidae) are -20.7 and -17.2°C (Milonas and Savopoulou-Soultani, 1999). If a candidate glasshouse biological control agent has the capacity to enter diapause, there is an increased probability of survival during a UK winter and in countries with a similar climate.

1.6. Thermal activity thresholds

Life processes such as predator avoidance, oviposition, foraging and prey consumption in a poikilothermic organism are bound by the thermal activity limits of the species (Ahnesjö and Forsman, 2006; Berger *et al.*, 2008; Crist and MacMahon, 1991; Gotoh *et al.*, 2004). Ideally, a candidate biological control agent should demonstrate foraging ability at a wide range of temperatures, and beyond the range of temperatures at which escape is possible by the pest.

Measurement of the ambulatory ability of flightless candidate biological control agents such as predatory mites will provide an indication of the foraging capacity of the species (Ding-Xu *et al.*, 2007). As poikilothermic locomotion is dependent on environmental temperature, a candidate biological control agent ideally should have the ability to forage at a greater range of temperatures than the target pest species has to evade predation.

1.6.1. CT_{min} and chill coma

The critical thermal minimum (CT_{min}) is a low temperature beyond which coordinated ambulation cannot occur, and has been described as a physiological event within the state of chill coma (Cowles & Bogert, 1944; Hazell and Bale, 2011). In a regime of decreasing temperature, chill coma is the point at which movement is no longer possible, and is often marked by a final spontaneous muscle action potential resulting in a limb twitch (Mellanby, 1939; Hosler *et al.*, 2000). Cessation of activity is likely to occur through the loss of ion regulation, which disrupts the excitability of muscle potential (MacMillan and Sinclair, 2011). In the short term, the state is non-lethal and movement is recoverable upon warming (Gilbert *et al.*, 2001).

Methods of measurement of the CT_{min} and chill coma have varied between the observation of knockdown temperature of insects in a glass column attached to a programmable alcohol bath; or the use of an arena milled into an aluminium block attached to a programmable alcohol bath (Fig. 1.6) (Kelty and Lee, 2001; Hazell *et al*., 2008). The aluminium block method has been described as more reliable as individual behaviour can be monitored continuously and without interference (Hazell *et al*., 2008; Hughes *et al*., 2010a).

1.6.2. Recovery

Chill coma is reversible, and individuals are able to regain use of their limbs (chill coma recovery) and ambulatory function (activity recovery) (Gilbert *et al.*, 2001; Hughes *et al.*, 2010a,b). The state of muscle paralysis is reversed as the homeostasis of haemolymph K^+ is recovered (MacMillan *et al.*, 2012). *Drosophila melanogaster* demonstrates a longer recovery time in individuals from higher temperature rearing regimes and prolonged cold exposures, highlighting chill coma recovery as an ecologically relevant measure of cold hardiness in arthropods (Macdonald *et al.*, 2004).

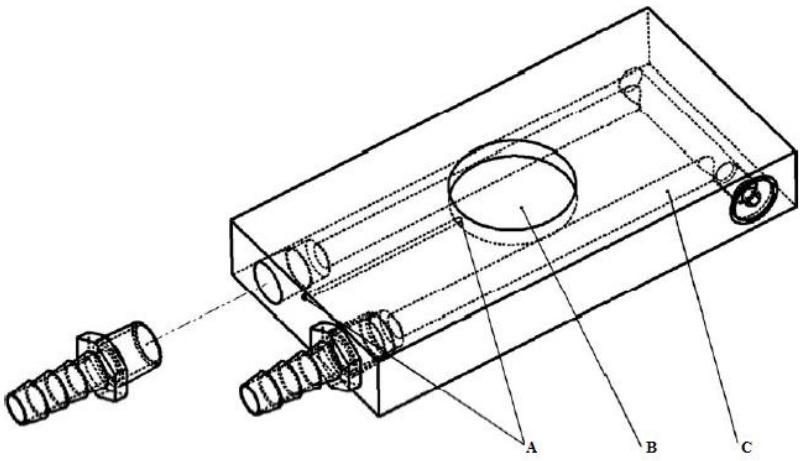

Figure 1.6. Arena milled into an aluminium block for the observation of invertebrate behaviour, A: passage for thermocouple; B: arena; C: channels for the flow of heated or cooled alcohol (from Hazell *et al*., 2008)

1.6.3. CT_{max} and heat coma

The critical thermal maximum temperature (CT_{max}) is a high temperature beyond which an arthropod cannot walk in a coordinated fashion (Cowles & Bogert, 1944). If the temperature is further increased following CT_{max}, movement of the antennae or legs will cease as the arthropod enters heat coma (Hughes *et al.*, 2010a,b). There is generally less variation in the upper thermal limits of invertebrates, which usually fall within the range of 40 to 50°C (Addo-Bediako *et al*., 2000). High temperature exposure degrades membranes, disrupting enzyme function and reducing the metabolism of the individual (Neven, 2000; Klok *et al*., 2001). Heat coma has been correlated with the upper thermal limit, and therefore is an irreversible state that will lead to the death of the individual (Hazell *et al.*, 2008).

1.7. Study species

Phytoseiulus macropilis and *B. hernandezi* are intended for inundative use in northern European glasshouses where internal conditions will support population growth. The thermal biology and thresholds of each candidate agent were investigated in this project to determine whether populations of glasshouse escapees could pose a risk of establishment. Ideally a candidate glasshouse biological control agent should be unable to survive typical winter conditions, limiting the impact of the non-native organism on the native environment (Hatherly *et al*., 2005a).

1.7.1. *Phytoseiulus macropilis* Banks

Phytoseiidae are used extensively as glasshouse biological control agents due to their highly effective control of *T. urticae* (McMurty & Croft, 1997). *Phytoseiulus persimilis* is currently a key phytoseiid biological control agent of *T. urticae*, and was applied to 8000ha of

commercial glasshouses worldwide by 1994 (Van Driesche and Bellows, 1996). *Phytoseiulus persimilis* is a spider mite specialist, and has a faster intrinsic rate of population increase compared to other commercially produced predators such as *N. californicus* (Escudero and Ferragut, 2005).

Phytoseiulus macropilis is a predatory phytoseiid mite with a native distribution in the Americas between 30°N and 15°S, and the Mediterranean (Fig. 1.7) (Shih *et al.*, 1979; Kreiter and de Moraes, 1997; de Moraes, 2004; Rosa *et al.*, 2005). The life cycle of *P. macropilis* consists of five developmental stages: egg, larva, protonymph, deutonymph and adult (Fig. 1.8). The species developmental response to temperature has been previously studied, and it was found that intrinsic rate of increase and net reproductive rate were highest at 28°C (Ali, 1998).

Figure 1.7. Adult female *P. macropilis* with on *T. urticae* webbing.

The species has been classified as a type I specialist predator, and as such will only predate spider mites (Croft *et al.*, 1998). Use of *P. macropilis* as a biological control agent has previously been investigated as the species is a more effective predator at higher temperatures compared to *P. persimilis* (Oliveira *et al.*, 2007). In glasshouse conditions at a predator: prey

ratio of 1:100, *P. macropilis* was able to bring a population of *T. urticae* on strawberry plants under control within 28 days (Oliveira *et al.*, 2009).

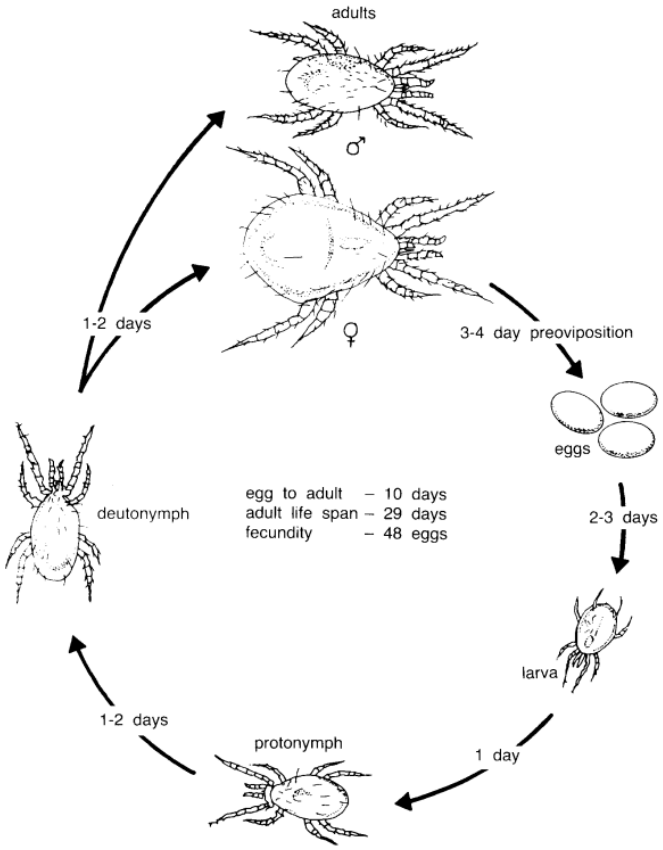

Figure 1.8. Life cycle of a typical phytoseiid mite at 27°C, 70% RH (from Yaninek *et al*., 1989)

1.7.2. *Balaustium hernandezi* Von Heyden

Balaustium hernandezi is a recently described species, originating from the Almeria region of Spain, with a life cycle consisting of seven instars: egg, prelarva, larva, protonymph, deutonymph, tritonymph and adult (Figs. 1.9, 1.10) (Mąkol *et al*., 2012). The species demonstrates regression in the protonymph and tritonymph stages, effectively alternating between active and quiescent stages, signifying a calypostasic life cycle (Belozerov, 2008a). Quiescent stages are formed beneath the cuticle of the previous instar, and will only emerge from the cuticulin layer upon ecdysis of the next active instar (Shatrov, 1999).

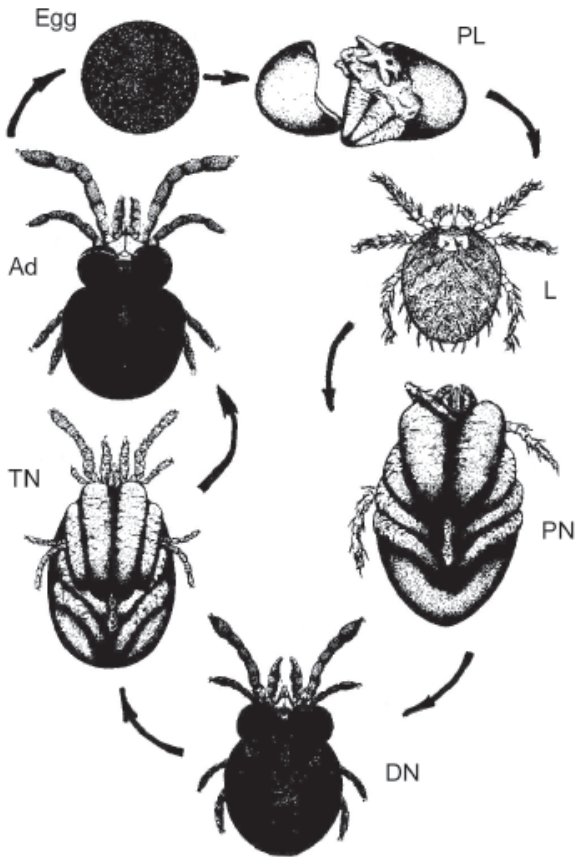

Figure 1.9. Life cycle of a species that undergoes alternating calyptostasy. PL: prelarva; L: larva; PN: protonymph; DN: deutonymph; TN: tritonymph; Ad: adult (from Belozerov, 2008a)

Figure 1.10. Adult female *B. hernandezi* on French bean surface with *T. urticae* females and eggs.

Thus far, only females have been identified in the laboratory populations of *B. hernandezi*, and it is hypothesized that the mites reproduce through parthenogenesis (Mąkol *et al*., 2012). Of twelve species studied within the genus, only *B. putmani*, *Balautium southcotti* Goldarenza and Zhang, and *Balaustium xerothermicum* sp. nov. (Acari: Erythraeidae) have male and female forms; only female individuals were identified in the remaining nine species (Gabryś, 2000; Mąkol *et al*., 2012). Parthenogenesis in invertebrates has been associated with polyploidy, which stabilises phenotypes in stressful environments (Otto and Whitton, 2000; Lencioni, 2004).

Balaustium hernandezi has urnulae, pitted tubule structures posterior to the eyes (Mąkol *et al*., 2012). The mites are able to secrete fluids from these structures, which have several defensive functions: release of alarm pheromones and allomones, and enhanced waterproofing (Yoder *et al.*, 2010; Yoder *et al*., 2007a). The waterproofing action of the urnulae derived fluids has been hypothesized to decrease the dehydration stress associated with high temperatures, allowing some species of the genus to survive exposures of 52°C (Yoder *et al*., 2007a).

Species within the genus *Balaustium* are generalists, and feed on the eggs and larvae of Tetranychids, Dipterans and Lepidopterans and pollen (Putman, 1970). These species have been proposed as ideal biological control agents, as a population would have longevity in a cropping system where target pest numbers were low, and may be able to suppress numerous pest species (Muñoz-Cárdenas *et al.*, 2013). However, this also poses a risk to other beneficial insects as intraguild predation, the consumption of competitor species, is associated with generalist diets (Polis *et al.*, 1989; Rosenheim *et al.*, 1993). Entomophagy is not ubiquitous in

the genus, as *B. medicanogoense* is an herbivorous crop pest in Australia, and some species have been reported to attack man (Arthur *et al*., 2011; Newell, 1963).

1.8. Objectives

The overall aim of this project is to determine whether two natural enemies of *Tetranychus urticae* are suitable for use in the UK as glasshouse augmentative biological control agents. The project will specifically focus on whether *Phytoseiulus macropilis* and *Balaustium hernandezi* are able to withstand a typical UK winter. The objectives are as follows:

1. To describe the relationship between temperature and survival of each predatory mite, and to produce estimates of various parameters of cold tolerance in the laboratory.
2. To compare laboratory and field results in order to assess the potential of the predatory mites to establish outside of the glasshouse environment, and compare acquired data with other previously studied biological control agents.
3. To characterise activity thresholds in response to chilling and warming.
4. To measure the predatory behaviour of candidate biological control agents which have the potential to be safely released for glasshouse biological control in the UK.

CHAPTER 2

Rearing Methods

The initial populations of all mite species were supplied by Biobest, NV (Belgium).

2.1. *Phytoseiulus macropilis* and *Phytoseiulus persimilis*

Each species was reared in quarantine at 25°C, 18:6h Light: Dark (LD). The rearing method was adapted from Hart *et al.* (2002b). The mites were placed on separate black ceramic tiles measuring 10 x 15cm which were outlined with Oecotak® (Oecos, UK) and placed on a sponge in a ventilated container half-filled with water, providing secure rearing arenas. 10 x 1cm strips of filter paper were placed across the tile and connected with the water in order to provide a moisture source for the mites. As male phytoseiids tend to die at onset of adverse conditions (Veerman, 1992), adult female mites were used for all experiments. The predatory mites were allowed to complete two generations in rearing conditions prior to use in experiments. The age of each cohort used were kept consistant: adults were removed for experiments within 7d of moulting from deutonymph; larvae were placed in experiments within 24h of egg hatch. To ensure larvae were less than 24h old, gravid females were removed from the rearing tiles and reared in test Eppendorfs (see section 2.2.4). Each female was provided with *T. urticae* individuals and checked for oviposition daily. Eggs were removed and placed in a seperate test Eppendorf, which was then checked every 24h for egg hatch, whereupon larvae were immediately removed and placed into experiments.

2.2. *Balaustium hernandezi*

Balaustium hernandezi Von Heyden (Acari: Erythraeidae) were cultured in a sealed container with dimensions 12 x 18 x 6 cm. The rearing boxes were half-filled with soil, and kept in quarantine at 25°C, 18:6 LD. The rearing boxes were sprayed daily with water until soil was moist to the touch, in order to provide a water source for the mites. Tritonymphs obtained from Biobest were allowed two weeks in rearing conditions prior to removal for use in experiments. This allowed time to moult, mate and acclimate to the rearing regime. The boxes were then retained in rearing conditions, and the soil sprayed daily until larval hatch.

2.3. *Tetranychus urticae*

Tetranychus urticae Koch (Acari: Tetranychidae) were reared as a food source for all predators on French bean plants, *Phaseolus vulgaris* L. (Fabaceae), in quarantine under 22 °C, 18:6 LD. *Tetranychus urticae* were transferred from damaged plants to fresh *P. vulgaris* weekly to maintain a large population. Infested *P. vulgaris* leaf discs were added daily to *Phytoseiulus macropilis* Banks and *Phytoseiulus persimilis* Athias-Henriot (Acari: Phytoseiidae) rearing tiles. Whole infested leaves were added to *B. hernandezi* rearing boxes each day, and removed and replaced the following day to prevent fungal growth.

2.4. Studying individual mites

As cannibalism is well documented within the Phytoseiidae and Erythraeidae, mites were studied individually in experiments monitoring mortality or egg-laying (Cadogan and Laing, 1977; Schausberger and Croft, 2000). 1.5ml Eppendorf ® tubes were selected to house mites individually during experiments. A hole with a diameter of 0.3cm was drilled into the tops of the tubes, and was then covered in 75µm mesh muslin to allow the flow of air. 0.5ml agar was

added to each Eppendorf to provide a moisture source for the mites throughout each experiment. *Tetranychus urticae* eggs and larvae were added daily over the course of each experiment to provide a source of food for the predators. This equipment will be referred to as a 'test Eppendorf' hereafter.

CHAPTER 3

Thermal biology of the spider mite predator *Phytoseiulus macropilis*

3.1. Abstract

Phytoseiulus macropilis Banks (Acari: Phytoseiidae) is a specialist predator of the two-spotted spider mite *Tetranychus urticae* Koch (Acari: Tetranychidae), and has been used to control the pest in its native range: Mediterranean regions, the tropics and Florida. This study investigates the thermal biology, as a proxy for establishment potential, of glasshouse escapees in cooler northern European climates, using a combination of laboratory and field trials. High mortality rates at 10°C indicated limited acclimation ability. Mites displayed continuous oviposition in conditions that have previously been shown to induce a hiatus in other Phytoseiid species, supporting previous findings that *Phytoseiulus* is a genus with no diapause state. Adult *P. macropilis* supercooled to between -17.2 to -24.0°C, but the $LTemp_{50}$ was -5.8°C, resulting in a high level of pre-freeze mortality. The $LTime_{50}$ at 5°C was 1.9 days, and maximum survival in winter field trials across 2010 and 2011 was 21 days. The thermal biology data indicate that *P. macropilis* is unlikely to establish in northern Europe, and will therefore make a suitable glasshouse biological control agent in temperate climates.

3.2. Introduction

Crop production in glasshouse structures is thought to cover 2.4 million hectares worldwide, and use in Europe has expanded over the past four decades (Pilkington et al. 2010; Hunt et al. 2008). Glasshouses offer a stable environment with controlled day lengths, irradiance, irrigation and temperature, allowing crop growth and yield to be advantageously manipulated

by commercial growers (Miller and Armitage 2002). The climate is controlled to promote maximum crop yield, consequently the conditions often correspond with an optimal environment for crop pest population growth. Traditionally, the expansion of a pest population was restricted using pesticides; however, the combination of increasing resistance, extensive non-target damage and consumer demand for pesticide-free produce has resulted in bans of many different compounds (Wilson and Tisdell 2001; Carvalho 2006). Biological control offers an effective solution to the control of crop pests that satisfies both the needs of the grower and the consumer.

Augmentative biological control is the application of a natural enemy in areas where abiotic factors prevent prolonged survival and reproduction (van Lenteren and Bueno, 2003). In Europe, augmentative biological control agents are applied effectively within the contained environment of a glasshouse, despite external ecosystem conditions preventing survival. In comparison to pesticide use, augmentative biological control is advantageous to growers as there are no phytotoxic repercussions for the plant, and no delay between application of the agent and harvest of the crop (van Lenteren 2000). The identification and development of biological control agents cost significantly less than chemical pesticides: £1.2 million compared with £112 million respectively (Bale et al. 2008). In more than 50% of instances where multiple biological control agents have been introduced, a single species has been responsible for the reduction of the pest population, demonstrating the system of biological control to be cost effective (Denoth et al. 2002). Over 7000 introductions of 2700 exotic invertebrates have been used to control pest species over the past century, and there have been very few reported deleterious impacts on the host ecosystem (Cock et al. 2010).

Predators, parasitoids and pathogens mass reared and distributed by commercial biological control companies in Europe have to adhere to the license requirements of the country of release. There are currently no harmonised EU directives concerning the introduction and regulation of exotic invertebrate natural enemies (Bigler et al. 2005; Bale 2011). The UK requires a license for release, consisting of an environmental risk assessment (ERA). However, several EU countries including Italy and Portugal have no regulations in place (Loomans 2007). Dispersal of introduced species within those countries without regulations will affect neighbouring countries, regardless of the policies the latter have in place.

There are several components of an ERA of a candidate augmentative biological control agent that require addressing prior to release. The host range of a candidate augmentative natural enemy must be narrow, and have limited dispersal ability from the area of release (van Lenteren et al. 2003). The agent should also lack cold hardiness that would otherwise allow escapees from the glasshouse environment to survive a typical winter (van Lenteren et al. 2003; Hatherly et al. 2005a). This is especially pertinent in northern Europe, where typical winter temperatures may act as a limiting factor on the growth of a poikilothermic population, and thus prevent a species from establishing and possibly becoming invasive. The cost of invasive non native species to the British economy is £1.7 billion (Williams et al. 2010). Conducting an ERA on a candidate augmentative biological control agent will reduce the chance of introducing a species that has the potential to become invasive.

This study is designed to investigate the survival and establishment potential of *Phytoseiulus macropilis* Banks (Acari: Phytoseiidae) in cooler northern European winters in countries such as the UK, the Netherlands and Scandinavia. The phytoseiid is found in Florida, the tropics

and Mediterranean regions (though it may have become naturalised in some of these areas), and is a successful predator of *Tetranychus urticae* Koch (Acari: Tetranychidae) in glasshouse conditions (Saba 1974; Moraes et al. 2004; Oliveira et al. 2007; Oliveira et al. 2008). *Tetranychus urticae,* known as the two-spotted spider mite or the glasshouse red spider mite, is a significant phytophagous pest species of many commercially grown flowers, vegetables and fruits (Driestadt et al. 2004). The data will indicate whether *P. macropilis* is safe for use in glasshouses of those northern European countries that require an ERA to be completed as part of the regulatory process.

3.3. Materials and Methods

3.3.1. Cultures and experimental procedures

Rearing methods of the mites are described in Chapter 2. Supercooling, lethal temperature, lethal time, diapause and field trial experiments have previously been used to assess the cold hardiness of several candidate biological control agents (Allen, 2010; Hughes *et al.*, 2009, 2011).

3.3.2. Acclimation

Ninety adults and 90 larvae were placed individually into test Eppendorfs, and supplied with *T. urticae* eggs and nymphs. These were randomly assigned to sealed ventilated boxes, and placed in an incubator set at 10°C 18:6 LD. One Tinytag ® datalogger was placed in each box to record the temperature at 10 min intervals throughout the trial. A fresh supply of *T. urticae* was transferred to the test Eppendorfs every 2 days.

Replicates of 30 adults and 30 larvae were sampled at 2, 4 and 7d. Eppendorfs were randomly removed from the boxes and transferred to rearing conditions. Mortality was assessed every 24h for the following 72h.

3.3.3. Supercooling point

The supercooling point of adult *P. macropilis* was assessed using methodology from Bale *et al*. (1984), and measured with a Pico TC-08 Thermocouple Data Logger. Individual mites were attached to type K thermocouples using a small volume of Oecotak and then placed in size three Beem capsules. The capsules were placed in boiling tubes suspended in a programmable alcohol bath. The temperature was be reduced at 0.5°C min^{-1} from rearing temperature to -30°C, and the supercooling point recorded as the temperature at which an exotherm was detected.

3.3.4. Lethal temperature

Individual *P. macropilis* were placed individually into size 3 Beem capsules. Five Beem capsules were loaded into a boiling tube, with 10 boiling tubes per treatment, and placed into a programmable alcohol bath. The temperature was then either reduced or increased from 25°C at 0.5°C min^{-1} to a range of temperatures predetermined by preliminary experiments to cause between 0 and 100% mortality. The mites were held at different exposure temperatures for 10 min and then warmed or cooled to rearing temperature at 0.5°C min^{-1}. For lower lethal experiments, adults and larvae were exposed to a range of temperatures between 5 and -10°C; in upper lethal experiments, mites were exposed to a range between 32 and 48°C.

Treated individuals were then placed separately into test eppendorfs, and held at rearing temperature. Mortality was recorded 72h after exposure.

A control treatment was carried out. Fifty mites were placed individually into size three Beem capsules, which were distributed between ten boiling tubes and placed into the alcohol bath. The mites were held at 25°C for the maximum experimental period (2.5h). Individuals were then placed into test Eppendorfs and held in rearing conditions. Mortality was recorded 72h after the exposure.

3.3.5. Lethal time

Phytoseiulus macropilis individuals were placed into size 3 Beem capsules with a strip of moist filter paper. Five Beem capsules were loaded into a 20ml glass vial, with 10 glass vials per treatment. All vials were held at 10°C for 1h to counter cold shock prior to immediately placing at 5, 0 or -5°C. Samples were removed at different time intervals determined by preliminary experiments, and held at 10°C for 1h to counter heat shock upon return to the culture temperature, 25°C. Individuals were then placed in a test Eppendorf, provisioned with *T. urticae* eggs and nymphs, and held in rearing conditions. Mortality was assessed 72h after the exposure.

Controls were placed into the same experimental conditions, but held at 25°C for the maximum experimental period (7d) prior to transfer to test eppendorfs.

3.3.6. Diapause

Eggs less than 12h old were taken from rearing conditions and placed on tiles at 15°C, 11:13 LD. The light regime was chosen as 11h is the critical day length for diapause induction in several phytoseiid species, including *Typhlodromips montdorensis* Schicha and *Metaseiulus*

occidentalis Nesbitt (Acari: Phytoseiidae) (Hoy, 1975; Hatherley *et al*., 2005b). The resulting population was allowed to mate and gravid females were placed individually into test eppendorfs. These were kept at 15°C, 11:13 LD and examined every day for oviposition, with the number of resulting eggs recorded. Any eggs laid in the 11:13 LD regime were reared through to adult in the same conditions. The resulting adults were allowed to mate to produce a second generation, which was also tested.

If, however, the females had not laid eggs within two weeks, they were transferred to original rearing conditions of 25°C, 18:6 LD. If oviposition occurred within five days following the transfer, it can be assumed the mites were in diapause. This period was chosen as phytoseiids that enter facultative diapause, such as *M. occidentalis*, will oviposit within five days following transfer from short to long day conditions (Hoy, 1975).

The results were compared with control samples. Thirty mites were placed at 15°C, 18:6LD and in rearing conditions (25°C, 18:6 LD) and monitored for oviposition.

3.3.7. Field trials

Mites were transferred into test Eppendorfs, and exposed to winter field conditions in four treatments: adults and larvae, both fed and unfed. Each treatment sample was allocated four replicates of ten mites. Fed treatments were supplied with *T. urticae* eggs and first and second instar nymphs. Test Eppendorfs were randomly assigned to one of four sealed ventilated boxes, and transferred to 10°C for 1h to counter mortality from cold shock. The boxes were then placed in a sheltered outdoor location at the University of Birmingham. One Tinytag ®

datalogger was placed in each box to record the temperature at 5 min intervals throughout the trial. A fresh supply of *T. urticae* was transferred to the fed treatments every five days.

Samples were removed from the field and transferred to 10°C for 1h to prevent mortality from heat shock, and *T. urticae* nymphs were added to the test eppendorfs. The Eppendorfs were then placed into rearing conditions and mortality was assessed after 24, 48 and 72h.

Winter 2010

Phytoseiulus macropilis were sampled at 1, 3, 5, 7, and 12d between 23rd November and 5th December 2010. Preliminary experiments indicated larvae were more likely to reach 100% mortality in a short time period and thus were sampled more frequently within the first seven days (1, 3, 5, 7d) compared to the adults (1, 3, 7d). Sufficient numbers of fed and unfed adults were used in order to allow sampling over a longer time period, as preliminary data demonstrated adults would not reach 100% mortality until between 14 and 28d.

Winter 2011

Seven samples of fed and unfed adult and larval *P. macropilis* were taken across 21d between 23rd November and 14th December 2011.

3.3.8. Statistical Analysis

Supercooling point data were analysed with a one-way ANOVA after determining the data were normally distributed. The results of lower lethal temperature and time experiments were analysed using SPSS 21 (IBM). Data were tested for normality using Pearson's Goodness-of-Fit test, and analysed using probit analysis (Finney 1971). Temperatures and times resulting

in 10, 50 and 90% mortality were selected from the output, and any differences between adult and larvae identified through a Parallelism Test, which tests whether each factor level has a common slope. The oviposition data were tested for normality and equal variances, and it was concluded that the non-parametric Kruskal-Wallis test was required. Further differences between the treatments were analysed using the Games-Howell *post-hoc* test, which does not assume equal variances. Field trial data were analysed using binary logistic regression to elicit differences between cohorts and feeding treatments. All results were considered significant when $p < 0.05$.

3.4. Results

3.4.1. Acclimation Ability

Fig. 3.1 demonstrates the mortality response of adult and larval *P. macropilis* exposed to an acclimation regime of 10°C 18:6 LD. Mean adult mortality slowly increased between days 2 and 7 (27 ± 7% and 43 ± 8% respectively). Larval mortality increased over the experimental period, reaching 97 ± 3% by day 7. As the acclimation regime elicited a relatively high mortality rate in both adults and larvae across the experimental period it was decided that an acclimation pre-treatment should not be used in other laboratory or field experiments.

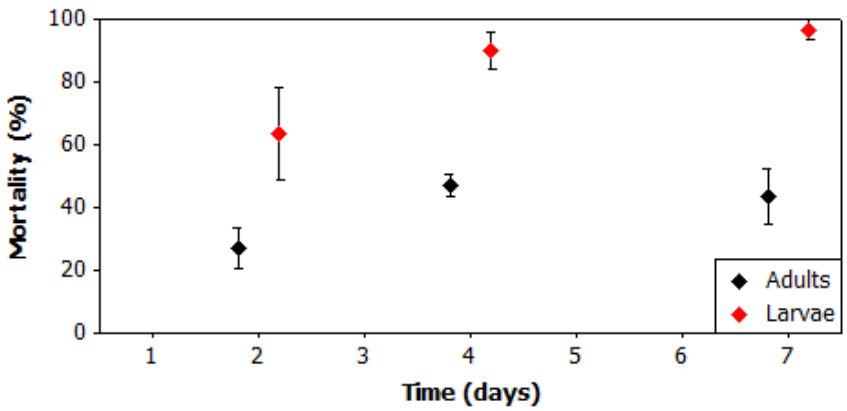

Figure 3.1. Mean (± SE) mortality of adult and larval *P. macropilis* at 10°C (n = 30).

3.4.2. Supercooling point

There was a significant difference of 1.6°C between the mean SCPs of adults and larvae (-21.7 ± 0.3 and -23.3 ± 0.2°C respectively) ($p < 0.001$; $F_{1,58} = 19.11$).

3.4.3. Lethal temperature

The probit estimates and 95% confidence intervals for lower and upper lethal temperature limits are displayed in Table 3.1. There was no mortality recorded in the control sample.

The upper lethal temperature data were normally distributed ($p = 0.715$; $\chi^2 = 4.551$; $df = 7$). Although the upper lethal temperature$_{10,50,90}$ of adults were higher than of the larvae, the Parallelism Test did not elicit a significant difference between cohorts ($p = 0.153$; $\chi^2 = 2.045$; $df = 1$) (Table 3.2).

Table 3.1 Parameter estimates of Probit analysis of upper and lower lethal temperatures

	Parameter	Cohort	Estimate	Std. Error	Z	Sig.	95% Confidence Interval	
							Lower bound	Upper bound
Upper lethal temperature[a]	Temperature		26.034	3.050	8.535	< 0.001	20.055	32.012
	Intercept	Adult	-41.327	4.868	-8.489	< 0.001	-46.195	-36.459
		Larvae	-41.109	4.860	-8.458	< 0.001	-45.969	-36.249
Lower lethal temperature[b]	Temperature		-3.730	0.317	-11.767	< 0.001	-4.351	-3.108
	Intercept	Adult	2.707	0.265	10.222	< 0.001	2.442	2.971
		Larvae	3.186	0.305	10.464	< 0.001	2.882	3.491

[a]PROBIT model: PROBIT(p) = Intercept + BX (Covariates X are transformed using the base 10.000 logarithm.)

[b]PROBIT model: (PROBIT(p) + 11) = Intercept + BX (Covariates X are transformed using the base 10.000 logarithm.)

The lower lethal temperature data were normally distributed (p = 0.118; χ^2 = 14.127; *df* = 9). The lower lethal temperature$_{10,50,90}$ of adults were higher than of the larvae, however, the result of the Parallelism Test indicated that there was no significant difference between adults and larvae (p = 0.087; χ^2 = 2.920; *df* = 1). The lower lethal temperature$_{50}$ was higher than the SCP for both cohorts.

Table 3.2 Estimated lethal temperatures resulting in 10, 50 and 90% mortality in adult and larval *P. macropilis*

	Cohort	Mortality (%)	Estimate (°C)	Std. Error	95% Confidence Interval	
					Lower bound	Upper bound
Upper lethal temperature	Adult	10	34.530	1.023	32.996	35.609
		50	38.674	0.005	37.822	39.465
		90	43.316	1.018	42.216	44.917
	Larvae	10	33.870	1.022	32.251	35.001
		50	37.936	1.012	37.034	38.722
		90	42.489	1.018	41.472	43.928
Lower lethal temperature	Adult	10	0.731	1.155	-0.755	2.936
		50	-5.683	1.086	-6.255	-5.090
		90	-8.590	1.249	-9.065	-8.154
	Larvae	10	4.776	1.132	2.703	7.852
		50	-3.849	1.083	-4.660	-2.998
		90	-7.758	1.236	-8.405	-7.160

3.4.4. Lethal time

The probit estimates and 95% confidence intervals for lethal time at 5, 0 and -5°C are shown in Table 3.3. The data were normally distributed in the 5°C (p = 0.125; χ^2 = 13.915; *df* = 9), 0°C (p = 0.128; χ^2 = 17.601; *df* = 12) and -5°C (p = 0.280; χ^2 = 9.796; *df* = 8) treatments. No mortality was recorded in the control treatment.

Table 3.3 Parameter estimates of Probit analysis of lethal time at 5, 0 and -5°C

	Parameter	Cohort	Estimate	Std. Error	Z	Sig.	95% Confidence Interval	
							Lower bound	Upper bound
Lethal time at 5°C[a]	Temperature		3.028	0.286	10.585	< 0.001	2.467	3.588
	Intercept	Adult	-1.265	0.173	-7.312	< 0.001	-1.438	-1.092
		Larvae	-0.781	0.152	-5.143	< 0.001	-0.932	-0.629
Lethal time at 0°C[a]	Temperature		1.060	0.090	11.720	< 0.001	0.883	1.237
	Intercept	Adult	-0.521	0.075	-6.948	< 0.001	-0.596	-0.446
		Larvae	-0.959	0.124	-7.719	< 0.001	-1.083	-0.834
Lethal time at -5°C[a]	Temperature		1.009	0.167	6.036	< 0.001	0.681	1.337
	Intercept	Adult	-0.325	0.079	-4.120	< 0.001	-0.403	-0.246
		Larvae	0.106	0.111	0.954	0.340	-0.005	0.217

[a]PROBIT model: PROBIT(p) = Intercept + BX (Covariates X are transformed using the base 10.000 logarithm.)

Larvae appeared to demonstrate 10, 50 and 90% mortality within shorter time frames than adults at 5 and -5°C. However, Parallelism Tests demonstrated that this difference was not significant at either 5°C ($p = 0.709$; $\chi^2 = 0.140$; $df = 1$) or -5°C ($p = 0.709$; $\chi^2 = 0.140$; $df = 1$). In contrast, larval survival was significantly prolonged at 0°C compared to adults ($p = 0.015$; $\chi^2 = 5.912$; $df = 1$).

Adult lethal $time_{10}$ at 0 and -5°C arose within an hour (0.2 and 0.1h respectively) with lethal $time_{50}$ following in reasonably quick succession (3.1 and 2.1h respectively). The lethal $time_{90}$ data were conservative compared to the lethal $time_{10,50}$ data. The observed 100% mortality points in each lethal time investigation for both cohorts were at maximums of 8 days at 5°C, 28h at 0°C and 10h at -5°C, which were all lower than the respective lethal $time_{90}$ probit results (Table 3.4). The probit estimate of larval lethal $time_{90}$ at 0°C was considerably higher

than observed maximum survival in experimental conditions (129.9 and 28h respectively), and was more prolonged than the lethal time$_{90}$ probit estimate of mortality at 5°C (115.2h).

Table 3.4 Estimated lethal time at 5, 0 and -5°C resulting in 10, 50 and 90% mortality in adult and larval *P. macropilis*

	Cohort	Mortality (%)	Estimate	Std. Error	95% Confidence Interval	
					Lower bound	Upper bound
Lethal time at 5°C (days)	Adult	10	0.987	1.263	0.722	1.234
		50	2.617	1.101	2.284	2.935
		90	6.935	1.189	5.986	8.429
	Larvae	10	0.683	1.278	0.489	0.873
		50	1.811	1.107	1.514	2.123
		90	4.799	1.230	3.995	6.054
Lethal time at 0°C (hours)	Adult	10	0.192	1.365	0.103	0.304
		50	3.101	1.152	2.381	4.045
		90	50.194	1.372	31.330	94.570
	Larvae	10	0.496	1.321	0.253	0.853
		50	8.024	1.167	5.078	13.049
		90	129.904	1.474	68.740	296.524
Lethal time at -5°C (hours)	Adult	10	0.113	1.765	0.031	0.223
		50	2.098	1.222	1.499	3.231
		90	39.066	2.090	16.409	203.982
	Larvae	10	0.042	2.471	0.007	0.112
		50	0.785	1.307	0.414	1.270
		90	14.623	1.804	7.308	49.687

3.4.5. Diapause

The mean number of eggs laid in different diapause and control regimes is shown in Fig. 3.2. The mean number of eggs laid per female per day was low in the lower temperature regimes, with females ovipositing less than once a day at 15°C 11:13LD and 15°C 18:6LD (0.47 and 0.88 eggs day^{-1} respectively). No mites were observed to suspend oviposition in the 11:13LD

treatments and then resume egg laying upon transfer to 18:6LD. Games-Howell *post-hoc* tests were run in order to determine which regimes produced significantly different numbers of eggs. The number of eggs laid by the first and second generations in short day conditions (15°C 11:13LD) did not significantly differ ($p = 0.999$). Despite some overlap on days 1 to 5 and day 10, the oviposition of the first and second generation in short day conditions significantly differed to that of the control (15°C 18:6LD) ($p < 0.001$). The number of eggs laid in the control regime was also significantly different to individuals in the rearing regime (25°C 18:6LD) ($P = 0.004$). The total number of eggs laid per regime was significantly different ($p < 0.001$; $H = 35.315$; $df = 3$).

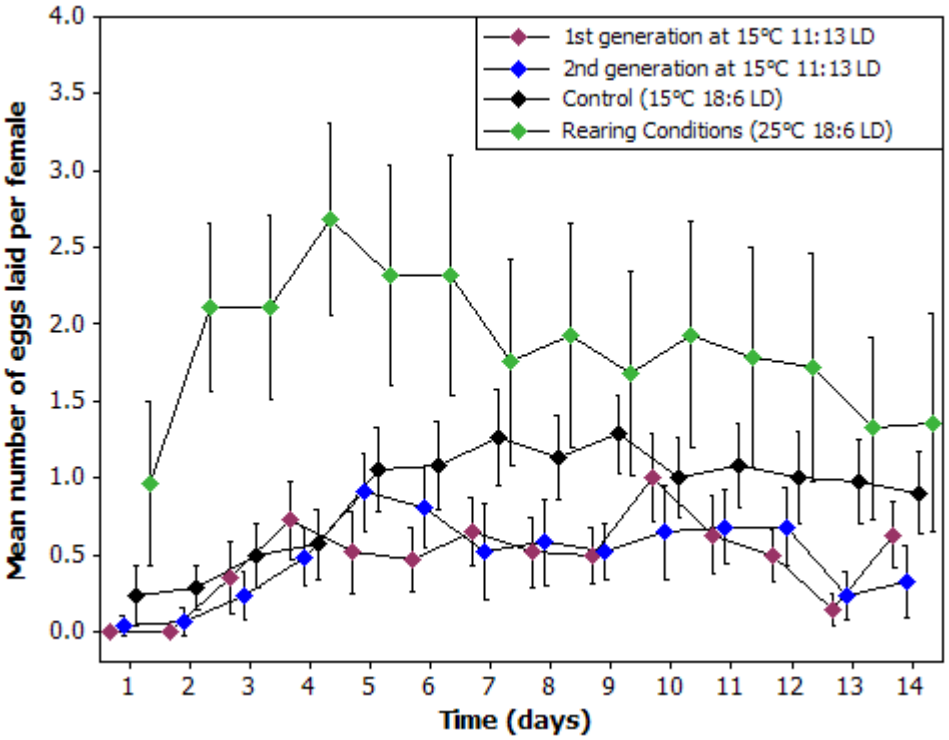

Figure 3.2. Mean (± SE) number of eggs laid by *P. macropilis* in different rearing regimes: 1st and 2nd generation at 15°C 11:13LD; control at 15°C 18:6LD and rearing conditions 25°C 18:6LD.

3.4.6. Field trials

Winter 2010

The minimum, mean and maximum temperatures and corresponding mortality of mites are shown in Fig. 3.3a and b. The minimum, mean and maximum temperatures of the sampling period were -6.0, -0.6 and 10.6°C respectively. All *P. macropilis* treatments reached 100% mortality by day 12. Binary logistic regression was used to elicit any significant differences between the cohorts and treatments, and data were assessed for goodness-of-fit using Pearson Chi-Square statistic ($p = 0.112$; $\chi^2 = 20.599$; $df = 14$). The mortality of adults and larvae was significantly different across the 12 days of the trial (Table 3.5). Interestingly, adult *P. macropilis* reached 100% mortality prior to the larvae, contrasting with preliminary field trials and laboratory measures of cold hardiness (Fig 3.3b). The fed *P. macropilis* treatments demonstrated significantly increased survival (Table 3.5).

Table 3.5 Parameter estimates of binary logistic regression of the 2010 and 2011 field exposures of fed and unfed adult and larval *P. macropilis*

Year	Parameter	Estimate	Std. Error	Z	Sig.	Odds Ratio	95% Confidence Interval	
							Upper Bound	Lower Bound
2010	Constant	0.502	0.360	1.39	0.163			
	Cohort	-0.997	0.214	-4.67	< 0.001	0.37	0.24	0.56
	Feeding Status	-0.872	0.204	-4.28	< 0.001	0.42	0.28	0.62
	Time	0.538	0.047	11.41	< 0.001	1.71	1.56	1.88
2011	Constant	-1.359	0.346	-3.93	< 0.001			
	Cohort	0.800	0.195	4.10	< 0.001	2.23	1.52	3.26
	Feeding Status	-0.656	0.194	-3.39	0.001	0.52	0.35	0.76
	Time	0.204	0.018	11.20	< 0.001	0.52	0.35	0.76

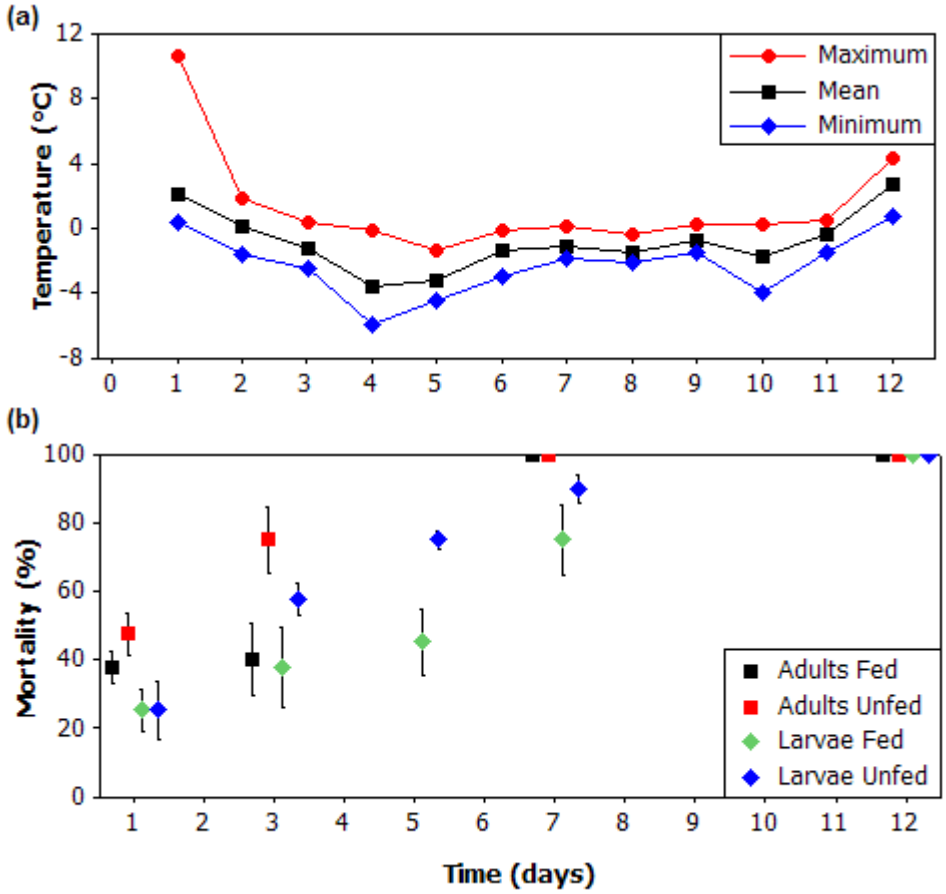

Figure 3.3. (a) Minimum, mean and maximum temperatures experienced by the adult and larval predatory mites and **(b)** mortality (mean ± SE) of fed and unfed adults and larvae in the field from 23rd November to 5th December 2010.

Winter 2011

The minimum, mean and maximum temperatures and corresponding mortality of mites are shown in Fig. 3.4a and b. The minimum, mean and maximum temperatures of the sampling period were -1.0, 5.7 and 11.6°C respectively. The mean temperature of the 2011 field trial was much higher than the 2010 field trial, and the investigation persisted over 21 days. Data were assessed for goodness-of-fit using the Hosmer-Lemeshow test ($p = 0.163$; $\chi^2 = 11.750$; $df = 8$). The mortality of adults and larvae was significantly different across the 21 days of the trial (Table 3.5). In contrast with the 2010 winter field trial, the larvae reached 100%

mortality by day 18, prior to the adults. Fed *P. macropilis* treatments demonstrated significantly increased survival (Table 3.5). Both the fed adults and larvae demonstrated lower mortality at each sampling period compared with their unfed counterparts (Fig. 3.4b). The first treatment to demonstrate 100% mortality was the unfed larvae at day 12.

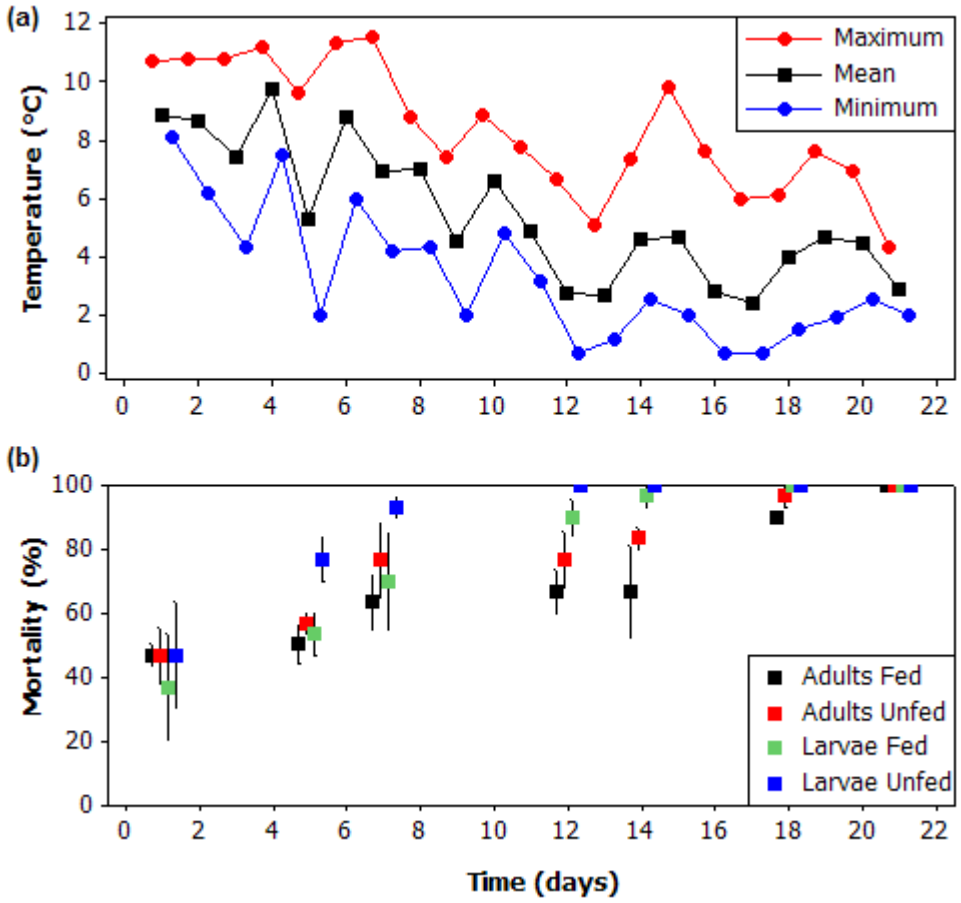

Figure 3.4. (a) Minimum, mean and maximum temperatures experienced by the adult and larval predatory mites and **(b)** mortality (mean ± SE) of fed and unfed adults and larvae in the field from 23rd November to 14th December 2011.

3.5. Discussion

Mites are poikilothermic, and therefore development, survival and establishment are affected by the temperature of the external environment. A combination of laboratory investigations

and field trials has previously been used to study the cold hardiness of a variety of different candidate augmentative biological control agents, including phytoseiid mites: *Neoseiulus californicus* McGregor (Hart et al. 2002b); *Typhlodromips montdorensis* (Hatherly et al. 2005b); *Amblyseius swirskii* Athias-Henriot and *Phytoseiulus longipes* Evans (Allen 2010). Ideally, *P. macropilis* used for glasshouse biocontrol should not demonstrate any robust cold hardiness, which would otherwise increase the likelihood of dispersal and establishment upon escape.

The results indicate that *P. macropilis* is unlikely to survive an acclimation regime prior to use in other cold hardiness experiments. The typical acclimation regime for previously studied biological control agents is 7 days at 10°C, as this is an adequate time period to prevent other extraneous factors influencing the results (Klok and Chown 2003). In the assessment of *A. swirskii* an acclimation regime of only 3 days at 10°C was used, as this was not deleterious to the adults or larvae, however mortality rose significantly by day 7 (Allen 2010). The high mortality of *P. macropilis* at day 3 rendered the use of an acclimation regime null, as the chilling injuries of any surviving mites would confound the results of any following experiments. Chilling injuries arise from the loss of body water at low temperatures, changing the haemolymph composition and altering the concentration of ions, which disturbs ion homeostasis processes (Koštál et al. 2004; Koštál et al. 2007). The results of these injuries are revealed by the inability of an arthropod to continue development or reproduce (Luczynski et al. 2007). The inability of *P. macropilis* to acclimate at 10°C indicates that the species would have limited ability to survive outside of a glasshouse during the onset of winter.

Mites are classed as a freeze intolerant taxon; consequently any freezing is lethal (Sømme 1999). Adult *P. macropilis* was able to depress its supercooling point to -21.7°C, thus avoiding the fatal effects of intracellular ice nucleation across the range of temperatures typically encountered in a temperate winter. However, the lower lethal temperature$_{50}$ of adult *P. macropilis* was -5.7°C, which is significantly higher than the supercooling point, and implies a high level of pre-freeze mortality. The combination of the supercooling point and lethal temperature data of *P. macropilis* identifies it as a chill-susceptible species (Bale 1993). This status renders the species comparable to other biological control agents such as *A. swirskii* (Allen 2010). *Amblyseius swirskii* has been deemed as a 'safe' augmentative biological control agent for northern Europe due to a lack of cold hardiness, and is now commercially available.

The upper lethal temperature data supports the use of the species as augmentative biological control agents in northern Europe. *Tetranychus urticae* infestations affect a variety of glasshouse plants, including crops such as tomato, *Solanum lycopersicum* Miller (Solanaceae) (Driestadt et al. 2004). Tomato crops are rarely exposed to temperatures above 30°C due to the decreased growth and metabolic rate, and rapid decline beyond 35°C (Criddle et al. 1997). The upper lethal temperature$_{50}$ of adult *P. macropilis* was 38.7°C, well above the optimum growth temperatures of tomato, and below the temperature at which the species loses the ability to maintain coordinated movement (Coombs and Bale 2013).

The lower lethal times were measured as a more ecologically relevant investigation of the cold tolerance of *P. macropilis*, where 5, 0 and -5°C represent typical mild, moderate and cold northern European winter temperatures respectively. The lethal time$_{50}$ (LTime$_{50}$) of adult *P.*

macropilis at 5°C was 2.6 days, rendering the species comparable with *A. swirskii* which had an $LTime_{50}$ of 2.7 days (Allen 2010). Use of the $LTime_{50}$ data indicates *P. macropilis* is a safe candidate for augmentative biological control, as if mites escape from the glasshouse, survival at even a relatively mild northern European winter temperature would be low.

Though the adult $LTime_{90}$ values at 5, 0 and -5°C were low (6.9 days, 50.2 and 39.1h respectively), the estimates seemed conservative compared to the 100% mortality observations made during the experiments (8 days at 5°C, 28h at 0°C and 10h at -5°C). Larvae were observed to perish prior to adults across all lethal time experiments, however the probit estimates of the $LTime_{50}$ at 0°C suggest an increased tolerance for low temperature exposures compared with adults (8.0 and 3.1h respectively). Reliability of probit estimates above 90% has previously been raised as a concern in a study of *Bactrocera tryoni* Froggatt (Diptera: Tephritidae) and *Epiphyas postvittana* Walker (Lepidoptera: Tortricidae) (Beckett and Evans 1997). However, as the values for $LTime_{90}$ are low, it is unlikely to affect the decision to class *P. macropilis* as safe for release.

Short day conditions at moderately low temperatures can induce diapause in some phytoseiid species including *Euseius finlandicus* Oudemans and *Neoseiulus fallacis* Garman (Acari: Phytoseiidae) (Broufas et al. 2006; Rock et al. 1971). However, mites originating from tropical and subtropical regions tend to show homodynamic development, and as yet, diapause has not been observed in the genus *Phytoseiulus* (Veerman 1992; Morewood 1993). Colder, short day conditions (11:13LD and 15°C) did reduce the oviposition rate of *P. macropilis*. This is likely to be related to temperature, as both low and high temperatures reduce the fecundity rate of poikilothermic arthropods, including mites such as *Tetranychus*

mcdanieli McGregor (Acari: Tetranychidae), *Euseius stipulatus* Athias-Henriot and *Typhlodromus phialatus* Athias-Henriot (Acari: Phytoseiidae) (Roy et al. 2003; Ferragut et al. 1987). Females oviposited less than once a day in short and long day conditions at 15°C, but there was a significant difference between the oviposition rates resulting from the treatments. An investigation of the oviposition rate of a non-diapausing strain of *Typhlodromus pyri* Scheuten (Acari: Phytoseiidae) found that 50% of individuals placed into 8:16 LD oviposited between 10 to 16 weeks, whereas 50% individuals in 18:6 LD oviposited within two weeks (Fitzgerald and Solomon 1991). As no *P. macropilis* females displayed a complete hiatus in egg production, the species seems unable to enter a diapause state, which again supports the view that the mite would be a safe species to release for glasshouse biocontrol in northern Europe.

The mean temperatures of the winter field trials in 2010 and 2011 differed significantly. Subzero temperatures across the majority of the 2010 field trial limited the survival of adult *P. macropilis* to 7 days, and larvae to 12 days. As the 2011 field trial was milder, and mites were not exposed to any subzero temperatures, *P. macropilis* reached 100% mortality at a slower rate. Maximum field survival in 2011 was 21 days, similar to *Nesidiocoris tenuis* Reuter (Heteroptera: Miridae) (23 days) and *Eretmocerus eremicus* Rose and Zolnerowich (Hymenoptera: Aphelinidae) (20 days), both of which have been classed as safe biological control agents in terms of their establishment potential (Hughes et al. 2009; Tullett et al. 2004).

The $LTime_{50}$ at 5°C is known to correlate with maximum survival of fed adults in the field, and can be used to identify candidate agents that pose low, medium or high risk of survival in

northern European winters (Hatherly et al. 2005a). The regression has been used to assess the likely field survival of the invasive *Harmonia axyridis* Pallas (Coleoptera: Coccinellidae), as well as several phytoseiid biological control agents: *A. swirskii* , *T. montdorensis*, *P. longipes*, and *N. californicus* (Raak-van den Berg et al. 2012; Allen 2010; Hatherly 2005a). Both *A. swirskii* and *T. montdorensis* fall within the 'low risk' category. Neither *P. longipes* nor *N. californicus* have been judged 'safe', and wild populations of *N. californicus* have been identified in the UK subsequent to applications in glasshouses (Allen 2010; Hatherly et al. 2005a; Jolly 2000). Despite using the more conservative estimate of 21 days survival from the winter 2011 field trial, *P. macropilis* can be identified as analogous to *A. swirskii*, as both duration of survival in the field over winter and $LTime_{50}$ at 5°C were low (Fig. 3.5).

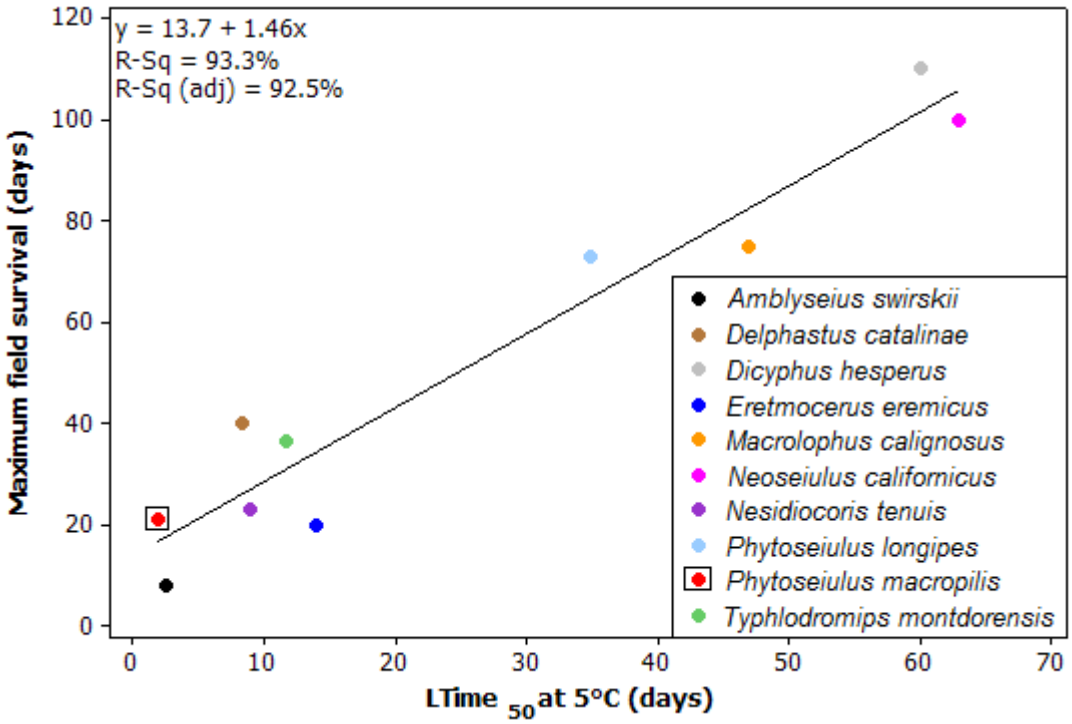

Figure 3.5. Correlation between $LTime_{50}$ at 5°C and maximum unfed field survival time of several adult biological control agents. Sources of data: *Amblyseius swirskii* (Allen, 2010); *Delphastus catalinae* (Tullett, 2002); *Dicyphus hesperus* (Hatherley *et al.*, 2008); *Eretmocerus eremicus* (Tullett *et al.*, 2004); *Macrolophus calignosus* (Hart *et al.*, 2002a); *Neoseiulus californicus* (Hart *et al.*, 2002b); *Nesidiocoris tenuis* (Hughes *et al.*, 2009); *Phytoseiulus longipes* (Allen, 2010); *Typhlodromips montdorensis* (Hatherley *et al.*, 2004).

The data acquired suggests the thermal biology of *P. macropilis* will prevent establishment outside of a glasshouse environment in northern Europe. The species cannot enter diapause and has a low tolerance of relatively mild winter conditions, implying a lack of cold hardiness that may otherwise aid survival, dispersal and establishment outside a glasshouse environment. An interesting question arises as to whether *P. macropilis* can survive year-round within protected environments. In glasshouses with continuous cultivation and *T. urticae* infestations, the predator would be able to maintain a long term population. However, in the absence of prey, and without the heating required for crop growth, a combination of starvation and cold-induced coma seems likely to prevent long term survival.

In a wider context, given that there is no likelihood of establishment outdoors by glasshouse escapees, consideration should be given as to whether *P. macropilis* and species with similar thermal biology profiles could be released in open field situations. Winter would act as a natural barrier to persistence from year to year, and whilst larger numbers would be present in the field with an 'open release', this would be a transient situation.

CHAPTER 4

Comparison of thermal activity thresholds of the spider mite predators *Phytoseiulus macropilis* and *Phytoseiulus persimilis*

4.1. Abstract

The lower and upper thermal activity thresholds of the predatory mite *Phytoseiulus macropilis* Banks (Acari: Phytoseiidae) were compared with those of its prey *Tetranychus urticae* Koch (Acari: Tetranychidae) and one of the alternative commercially available control agents for *T. urticae*, *Phytoseiulus persimilis* Athias-Henriot (Acari: Phytoseiidae). Adult female *P. macropilis* retained ambulatory function (CT_{min}) and movement of appendages (chill coma) at significantly lower temperatures (8.2 and 0.4°C respectively) than that of *P. persimilis* (11.1 and 3.3°C) and *T. urticae* (10.6 and 10.3°C). As the temperature was raised, *P. macropilis* ceased walking (CT_{max}) and entered heat coma (42.7 and 43.6°C respectively), beyond the upper locomotory limits of *P. persimilis* (40.0 and 41.1°C), but before *T. urticae* (47.3 and 48.7°C). Walking speeds were investigated and *P. persimilis* was found to have significantly faster ambulation than *P. macropilis* and *T. urticae* across a range of temperatures. The lower thermal activity threshold data indicate that *P. macropilis* will make an effective biological control agent in temperate climates.

4.2. Introduction

An invertebrate biological control agent must be both safe and effective in order to merit release (McClay and Balciunas, 2005). Several organisations have collaborated and developed a framework of information required to assess the safety of a potential invertebrate biological

control agent (OECD, 2004; Bigler *et al.*, 2005a,b; van Lenteren *et al.*, 2006). In order to comply with these guidelines, a potential biological control agent must be assessed using methodologies to determine establishment potential, host range and dispersal as part of an environmental risk assessment (van Lenteren *et al.*, 2003; van Lenteren *et al.*, 2006). This approach has successfully been used to identify the first classical weed biological control agent for the EU: *Aphalara itadori* Shinji (Hemiptera: Psyllidae) to control Japanese knotweed, *Fallopia japonica* Houtt (Polygonaceae) (Shaw *et al.*, 2009). Laboratory and field trials examining the likelihood of establishment are necessary for both classical and augmentative biological control agents, to ensure or prevent establishment respectively. As the augmentative approach is the prevalent form of biological control in Europe, previous investigations have concentrated on abiotic factors, such as temperature, as the basis for the prevention of the establishment of non-native natural enemies (Tullett *et al.*, 2004; Hatherley *et al.*, 2005; Bale, 2008; Hughes *et al.*, 2009). The examination of lower thermal activity thresholds indicates whether a candidate glasshouse biological control agent will have the ability to disperse away from a glasshouse environment during typical winter conditions.

Effective control is a fundamental consideration in the selection of an invertebrate biological control agent. The efficacy of the agent will, in part, be determined by its thermal thresholds, which dictate the lowest or highest temperature at which activities such as foraging or reproduction are possible. The critical thermal minima (hereafter referred to as 'CT_{min}') is defined as the lowest temperature at which coordinated walking can occur (Cowles & Bogert, 1944). If the temperature falls further beyond this point, movement of the appendages will cease as the individual enters chill coma (Mellanby, 1939). Provided that the temperature does not remain below the chill coma threshold for a prolonged period, invertebrates can recover

both movement of limbs and the ability to walk ('chill coma recovery' and 'activity recovery') as temperature rises. The ideal biological control agent would enter and recover from CT_{min} and chill coma at a lower temperature than its target prey or host, allowing movement and foraging behaviour whilst the pest remains immobile.

Similarly, upper thermal thresholds consist of the critical thermal maxima ('CT_{max}'), a high temperature beyond which an individual cannot walk in a coordinated fashion (Cowles & Bogert, 1944). If the temperature continues to rise, movement of appendages will cease as the invertebrate enters heat coma. Previous studies have shown that heat coma coincides with the upper lethal limit, and invertebrates cannot recover from this exposure (Hazell *et al.*, 2010; Hughes *et al.*, 2010a,b). There is thought to be little variation in the upper lethal temperature of invertebrates, usually falling within the range of 40 and 50°C (Heinrich, 1981); as with low temperatures, it would be beneficial if the biological control agent were to remain active at temperatures above the upper activity thresholds of the target pest.

This investigation concerns the thermal thresholds of a predatory mite, *Phytoseiulus macropilis* Banks (Acari: Phytoseiidae). *P. macropilis* is found in the tropics, the US and the Mediterranean, and is a successful predator of *Tetranychus urticae* Koch (Acari: Tetranychidae) in glasshouse conditions (de Moraes *et al*, 2004; Oliveira *et al*., 2007; Oliveira *et al.*, 2009). The species is now being investigated for use as an augmentative biological control agent in northern European countries where an environmental risk assessment is required as part of a license for release. *Phytoseiulus persimilis* Athias-Henriot (Acari: Phytoseiidae) has been used as an augmentative biological control agent of *T. urticae* since 1968, and is currently applied in twenty countries (Cock *et al*., 2010). The thermal activity

thresholds and walking speed of each species are compared in order to indicate whether *P. macropilis* will be an effective control agent across a range of temperatures. Ideally, the range of temperatures at which *P. macropilis* is active should be broadly equivalent to those of *P. persimilis* and *T. urticae* in terms of its activity range (lower and upper thresholds), as well as speed of movement.

4.3. Materials and Methods

4.3.1. Cultures

Rearing methods of the mites are described in Chapter 2.

4.3.2. Experimental System

The experimental system developed by Hazell *et al.* (2008) has previously been used to assess the thermal activity thresholds of two phytoseiids: *Phytoseiulus longipes* Evans and *Amblyseius swirskii* Athias-Henriot (Allen, 2010), as well as a variety of other invertebrate species (Hazell *et al.*, 2010; Hughes *et al.*, 2010a,b). Mites were placed in an arena with a diameter of 16mm and a depth of 7.5mm in an aluminium block that allowed the passage of cooled or heated fluids from a connected alcohol bath (Haake Phoenix 11 P2, Thermo Electron Corp., Germany). The arena was covered with a clear plastic petri dish, and the walls of the arena coated with Fluon (Blades Biological, UK) to prevent the escape of the mites. A type K thermocouple connected to a thermometer (Tecpel Advanced Digital Thermometer DTM-315, Heatmiser, UK) was inserted into the arena wall, which recorded the temperature throughout the experiment.

The thermal activity thresholds of the mites were recorded for retrospective analysis using an Infinity 1 digital camera (Lumenera, Ottowa, Canada), with a 10x macro lens (MLH-10X, Computar, CBC Corp., New York, USA). Videos were recorded using Studio Capture, and analysed using Studio Player and Studio Measure (Studio86Designs, Lutterworth, UK).

4.3.3. Calibration

CT_{min}, chill coma and recovery experiments were conducted in a controlled environment room at 10˚C. CT_{max} and heat coma experiments were conducted in a similar room at 23˚C. Though the aluminium block serves as a conductor for the temperature of the alcohol flowing within it, the temperature within the arena varies spatially due to the external temperature (Piyaphongkul, 2013). In order to negate the difference in temperatures experienced by the mites according to their location in the arena, a calibration was undertaken. Three replicates of 5 mites were attached to type K thermocouples using Oecotak® and placed in different locations in the arena. The temperature of the arena used for lower thermal thresholds was reduced from 25 to -12˚C, and the arena used for upper thermal thresholds was increased from 25 to 55˚C. The temperatures of each of the 5 mites were recorded at regular intervals. The results were compared with the temperature recorded by the thermocouple in the wall of the arena using linear regression. All experimental results were then calibrated using the results of the linear regression.

4.3.4. Body Size Measurement

Ten individual adult females of each species were placed into the arena, and recorded for 5s. One frame of the video was selected, and the length of the idiosoma was measured.

4.3.5. CT_{min} and Chill Coma

A sample of six mites were transferred from rearing conditions into the arena, and the temperature was reduced at a rate of 0.2°C min^{-1} from 25 to -2°C, as preliminary experiments demonstrated 100% of *P. macropilis, P. persimilis* and *T. urticae* entered chill coma prior to this temperature. Previous investigations have demonstrated that the slower the rate of cooling, the lower the CT_{min}; as slower cooling rates are more ecologically relevant a cooling rate of 0.2°C min^{-1} was adopted (Nyamukondiwa and Terblanche, 2010). CT_{min} was recorded as the temperature at which each individual made a final coordinated ambulatory movement, and chill coma as temperature at which the final twitch of an appendage was made. Thirty individuals of each species were monitored for entry into CT_{min} and chill coma.

4.3.6. Chill Coma and Activity Recovery

Thirty fresh individuals of each species were used to measure chill coma and activity recovery. Samples of six mites were transferred from rearing conditions into the arena, and the temperature was reduced from 25 to -2°C at a rate of 0.5°C min^{-1}. The arena was held at -2°C for 10 min to ensure all individuals had entered chill coma, and then returned back to 25°C at a rate of 0.2°C min^{-1}. As the temperature increased, chill coma recovery was recorded as the temperature of the earliest twitch of an appendage, and activity recovery as the temperature at which the mite resumed coordinated locomotion.

4.3.7. CT_{max} and Heat Coma

Thirty individuals of each species were observed for entry into CT_{max} and heat coma. Samples of six mites were transferred from rearing conditions into the arena, and the temperature was increased from 25 to 55˚C at a rate of 0.2°C min^{-1}. CT_{max} was measured as the temperature at

which each individual made a final coordinated ambulation, and heat coma as the temperature at which the final twitch of an appendage was made. As entry into heat coma was effectively the upper lethal temperature, it was not possible to record recovery from heat coma.

4.3.8. Walking Speed

Thirty individuals of each species were observed to determine the mean walking speed at a range of temperatures. Samples of three mites were transferred from rearing conditions into the arena. The temperature of the arena was raised from 25 to 30°C, and individuals were recorded for 10 min. The temperature of the arena was then lowered at 5°C intervals to 0°C at 0.5°C min^{-1}, holding the mites at each progressively lower exposure temperatures for 10 min. The first 5 min of each exposure were allocated in order to negate any lag in temperature between the arena and the mites, therefore only the final 5 min of each exposure was analysed. The distance each mite travelled over the 5 min was measured, from which mean walking speed could be calculated.

Videos were analysed using Studio Measure, which allowed measurements to be made on a frame-by-frame basis. As there was minimal movement of mites between frames, issues presented by ambulation on an irregular path were negated, as circular or indirect motion could be accurately measured and accounted for.

4.3.9. Statistical Analysis

All statistical analyses were made using the statistical package Minitab 15 (Minitab Ltd., Coventry, UK).

The critical thermal minima and maxima, entry into chill and heat coma and resumption of activity were initially analysed using distribution ID plots, which confirmed the most appropriate distribution to utilise in further analysis of the results. In all cases the Weibull distribution was considered the best fitting and therefore most suitable, and is a commonly utilised distribution in the analyses of life data (Clifford Cohen, 1965). Upon ascertaining that the data were normally distributed, parametric distribution analyses could be performed using the Weibull distribution. These analyses identify differences in both shape and scale of the data between each species. Significant differences within the data were confirmed using one way ANOVAs and Tukey's HSD *post-hoc* tests.

The walking speed data were first tested for normality and equal variances, and it was determined that a non-parametric test was required. The data were analysed using the Scheirer-Ray-Hare extension of the Kruskal-Wallis test.

Results were considered significant when $p < 0.05$.

4.4. Results

4.4.1. Calibration

The difference between arena temperature and actual mite temperature varied between species and arena, and the resulting regression equations were used to analyse corresponding data (Fig. 4.1).

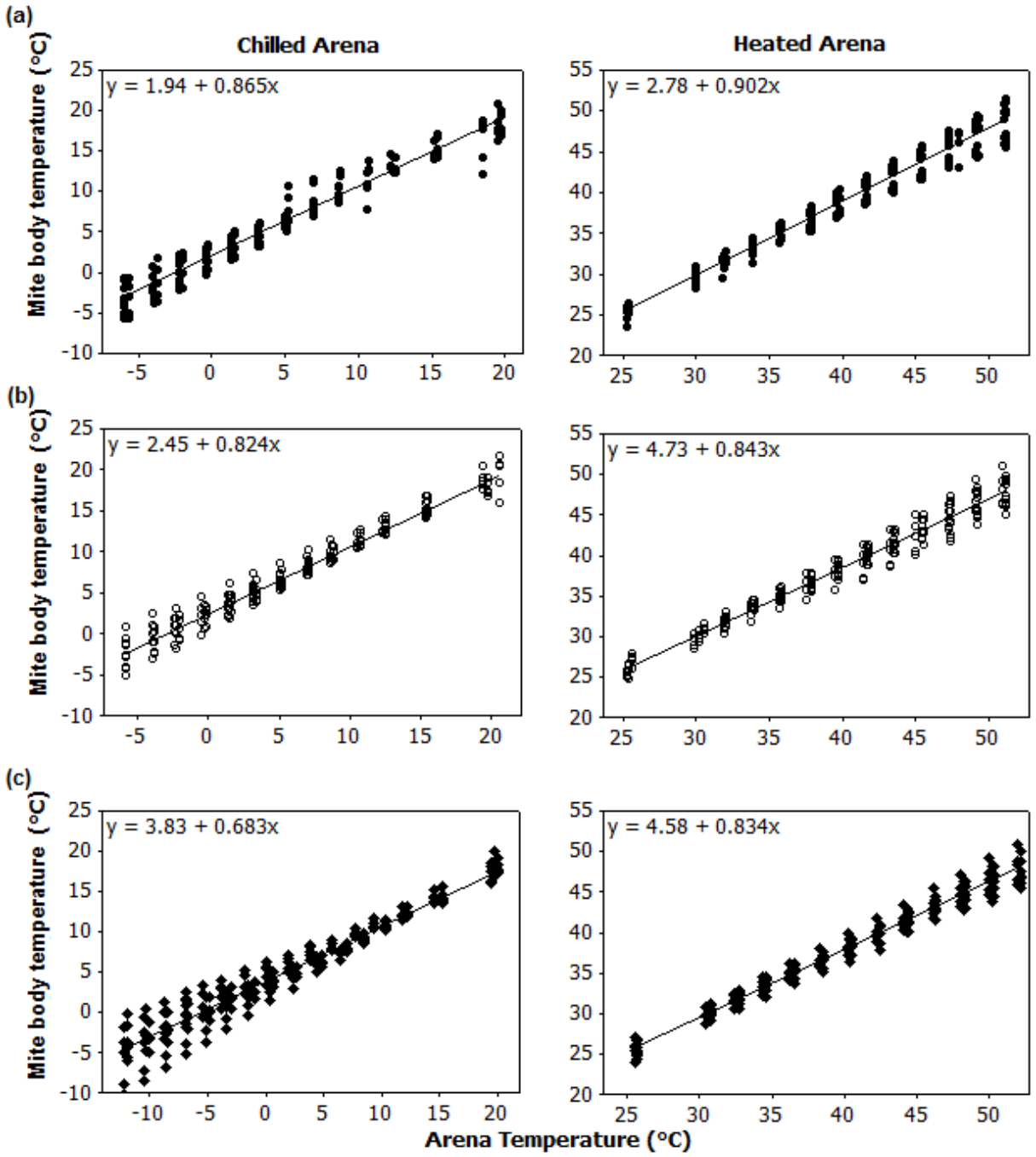

Figure 4.1. Regressions of arena temperature against body temperature for the calibration of lower and upper thermal thresholds in the chilled and heated arenas respectively. Calibrations were undertaken for **(a)** *P. macropilis*, **(b)** *P. persimilis* and **(c)** *T. urticae*.

4.4.2. Body Size Measurement

Tetranychus urticae had the largest mean idiosoma of the three species (420 ± 25μm). The mean idiosoma length of *P. persimilis* was slightly larger than *P. macropilis* (325 ± 16μm and 310 ± 11μm respectively).

4.4.3. CT_{min} and Chill Coma

There was a significant difference between the mean temperatures at which the two predators and *T. urticae* lost ambulatory function ($p < 0.001$; $\chi^2 = 24.00$; $df = 4$). *Post-hoc* tests revealed that there was no difference between the mean temperatures at which *P. persimilis* and *T. urticae* reached CT_{min} (11.1 and 10.6°C respectively); however, *P. macropilis* retained ambulatory function to a significantly lower mean temperature (8.2°C) (Table 1). The range of temperatures at which *T. urticae* and *P. persimilis* experienced CT_{min} was greater than those of *P. macropilis* (Table 4.1).

The mean chill coma temperature also significantly differed between species ($p < 0.001$; $\chi^2 = 208.56$; $df = 4$). There was no significant difference between the mean CT_{min} and chill coma temperatures of *T. urticae* ($p = 0.610$; $F_{1,58} = 0.26$); however both *P. macropilis* and *P. persimilis* maintained use of their appendages to a significantly lower temperature than their CT_{min} (0.4 and 3.3°C respectively). Tukey's HSD *post-hoc* test showed that *P. macropilis* entered chill coma at a significantly lower temperature than *P. persimilis* ($p < 0.001$). *Phytoseiulus macropilis* also encountered chill coma at a narrower range of temperatures than those of both *P. persimilis* and *T. urticae* (Table 4.1).

Table 4.1 Mean ± SE and range (in brackets) of temperatures (°C) at which each species experienced CT_{min}, chill coma, chill coma recovery, and activity recovery. Means followed by different letters are significantly different (Tukeys HSD, $p < 0.05$)

Species	CT_{min}	Chill coma	Chill coma recovery	Activity recovery
Phytoseiulus macropilis	8.2 ± 0.4 [a] (4.9 – 12.9)	0.4 ± 0.2 [c] (-1.0 – 3.4)	2.9 ± 0.2 [e] (1.5 – 6.9)	16.4 ± 0.3 [g] (13.7 – 20.8)
Phytoseiulus persimilis	11.1 ± 0.6 [b] (5.5 – 17.0)	3.3 ± 0.5 [d] (-0.6 – 9.4)	2.2 ± 0.6 [e] (-0.1 – 12.4)	16.8 ± 0.5 [g] (11.9 – 21.8)
Tetranychus urticae	10.6 ± 0.5 [b] (5.3 – 16.2)	10.3 ± 0.5 [b] (4.7 – 15.7)	12.2 ± 0.6 [f] (8.4 – 20.4)	12.8 ± 0.6 [f] (8.4 – 20.4)

4.4.4. Chill Coma and Activity Recovery

The shape and scale of the distribution of chill coma recovery temperatures differed significantly between species ($p < 0.001$; $\chi^2 = 1986.72$; $df = 4$), although examination of Tukey's HSD *post-hoc* tests revealed no difference between the two phytoseiids (Table 4.1). Chill coma recovery occurred at a significantly higher mean temperature in *T. urticae* (12.2°C) than *P. macropilis* and *P. persimilis* (2.9 and 2.2°C respectively).

Mean activity recovery temperature was also significantly different between the three species ($p < 0.001$; $\chi^2 = 46.44$; $df = 4$). There is a pronounced difference between the mean chill coma recovery and activity recovery of the phytoseiids compared with *T. urticae* (Table 4.1). Whilst *P. macropilis* entered CT_{min}, chill coma and recovered from chill coma at lower temperatures than *T. urticae*, it is notable that the predator resumed walking at a significantly higher temperature ($p < 0.001$; $F_{1,58} = 24.09$). There was, however, no significant difference between the mean temperature of activity recovery of *P. macropilis* and *P. persimilis* (16.4 and 16.8°C respectively).

The ranges of both chill coma recovery and activity recovery temperatures are greater in *T. urticae* and *P. persimilis* than *P. macropilis* (Table 4.1).

4.4.5. CT_{max} and Heat Coma

There was a significant difference between the temperatures at which each species reached CT_{max} ($p < 0.001$; $\chi^2 = 101.82$; $df = 4$). *Tetranychus urticae* maintained coordinated walking until a mean temperature of 47.3°C, beyond the temperatures at which both *P. persimilis* (40.0°C) and *P. macropilis* (42.7°C) ceased movement. The range of temperatures at which each predator entered CT_{max} was narrower than those of *T. urticae*; *P. persimilis* reached CT_{max} across a more limited range of temperatures than *P. macropilis* (Table 4.2).

Table 4.2 Mean ± SE and range (in brackets) of temperatures (°C) at which each species experienced CT_{max} and heat coma. Means followed by different letters are significantly different (Tukeys HSD, $p < 0.05$)

Species	CT_{max}	Heat coma
Phytoseiulus macropilis	42.7 ± 0.5 ^{h} (35.4 – 46.9)	43.6 ± 0.5 ^{h} (36.6 – 48.0)
Phytoseiulus persimilis	40.0 ± 0.4 ^{i} (35.1 – 43.3)	41.1 ± 0.4 ^{i} (35.3 – 45.1)
Tetranychus urticae	47.3 ± 0.9 ^{j} (39.4 – 54.9)	48.7 ± 0.7 ^{j} (43.8 – 55.2)

Although there was a difference between the three species, the mean temperature at which *T. urticae* entered heat coma was not significantly different to its mean CT_{max} ($p = 0.251$; $F_{1,58} = 1.35$). Likewise, there was no significant difference between the mean temperature at which CT_{max} and heat coma were encountered by *P. macropilis* ($p = 0.202$; $F_{1,58} = 1.66$) or *P. persimilis* ($p = 0.104$; $F_{1,58} = 2.72$) (Table 4.2).

The mean heat coma temperature differed significantly between species ($p < 0.001$; $\chi^2 = 130.69$; *df* = 4). The mean heat coma temperature was highest in *T. urticae* (48.7°C), significantly higher than that of either *P. macropilis* (43.6°C) or *P. persimilis* (41.1°C) ($p < 0.001$; $F_{1,87} = 47.41$). *T. urticae* entered heat coma across a wider range of temperatures compared with either predator (Table 4.2).

4.4.6. Walking Speed

There was a significant difference between the walking speed of each species at all temperatures ($p < 0.001$; $H = 46.60$; *df* = 2). However, this can be attributed to the walking speed of *P. persimilis*, which is much faster than *P. macropilis* and *T. urticae* at all temperature intervals between 10 and 30°C (Figure 4.2). Though there was an apparent disparity in walking speeds of *P. macropilis* and *T. urticae* at 5, 10, 15 and 20°C, there was no significant difference between the two species ($p = 0.438$; $H = 0.56$; *df* = 1). Although *P. macropilis* appears to have a faster mean walking speed than *T. urticae* at these intervals, comparisons show overlapping of the Bonferroni 95% confidence intervals between the two species at all temperatures.

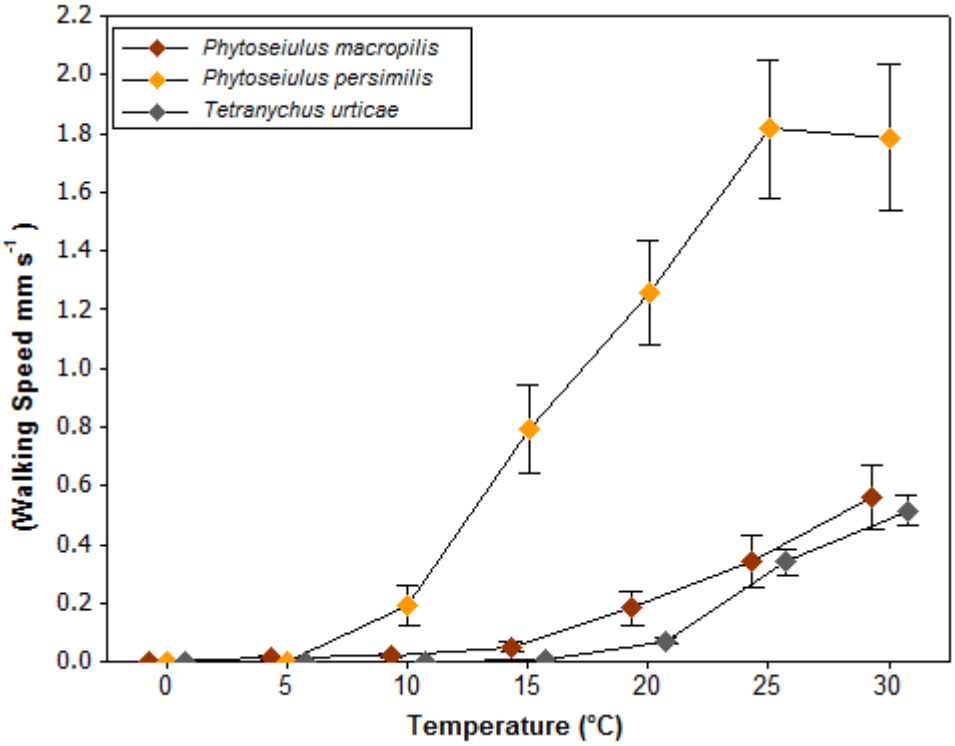

Figure 4.2. Mean (± SE) walking speed (mm s^{-1}) of *P. macropilis*, *P. persimilis* and *T. urticae* at different temperature intervals.

4.5. Discussion

Consideration of thermal activity thresholds is an important component in the examination of the thermal biology of invertebrates (Hughes *et al.*, 2010a,b; Hazell and Bale, 2011; MacMillan and Sinclair, 2011). The efficacy of a potential biological control agent will be demonstrated by thermal thresholds, as activity at extreme low and high temperatures compared to the prey species characterises the extent to which the agent will be effective. Ideally an augmentative biological control agent intended for use in a temperate region, such as *P. macropilis*, should remain active at temperatures at which its target prey become immobile.

Phytoseiulus macropilis retained locomotor function below the temperature at which both *T. urticae* and *P. persimilis* became immobile. This result suggests that *P. macropilis* would be an effective augmentative control agent in temperate climates, as the predator remains active at temperatures below which the pest is unable to move. *Phytoseiulus macropilis* therefore has the potential to forage whilst the prey is unable to take evasive action, and *P. persimilis* is less able to compete.

There is a difference, albeit small, in the mean size of adult female *P. macropilis,* compared with the larger adult female *P. persimilis* and *T. urticae*. The disparity in size may account for *P. macropilis* having lower thermal activity thresholds than *P. persimilis* and *T. urticae*, as CT_{min} has been shown to correlate with surface area to volume ratio ('SVR') in other arthropods, where species with the highest SVR can maintain activity at lower temperatures than those with lower SVR (Renault *et* al., 2003; Le Lann *et al*., 2011).

Interestingly, despite having a lower chill coma recovery than *T. urticae,* the activity recovery of both predators is at a significantly higher temperature than the pest. The phytoseiid *A. swirskii* demonstrated a similar response to *P. macropilis* and *P. persimilis*, with some individuals resuming ambulation at temperatures as high as 25˚C (Allen, 2010). Chill coma arises from the disruption of both enzyme activity and fluidity of the phospholipid bilayer (Hazel, 1995; Fields, 2001). Chill coma recovery is thought to be the reversal of these chilling injuries; however, the underlying mechanisms remain unclear (Nilson *et al.,* 2006). As recovery of coordinated walking depends on restored functioning of the metabolism, the magnitude and longevity of chilling injuries sustained during chill coma will have a direct bearing on the activity recovery time.

Upper thermal limits of invertebrates show much less variation than lower limits (Addo-Bediako *et al.*, 2000). Although the upper thermal activity thresholds significantly varied between the three species studied, there was no difference between the CT_{max} and heat coma within each species. As the temperature increases, deleterious modifications occur within the enzymes and membranes which alter the metabolism of the individual (Neven, 2000). Such lethal modifications can occur due to a decrease in pH, as the cytosol is known to increase in acidity at approximately -0.017 pH units per 1°C rise in temperature (Hochachka and Somero, 1984). Increased acidity will irreversibly damage nucleic acids and proteins. Using heat coma as a proxy for the upper lethal limit, *P. macropilis*, *P. persimilis* and *T. urticae* fall within the expected range of 40 to 50°C for maximum temperature of invertebrate survival (Heinrich, 1981; Hazell *et al.*, 2010). As the predators reach the upper thermal activity threshold at a lower temperature than *T. urticae*, the efficacy of each phytoseiid will not be as pronounced in environments where temperatures reach 40°C or higher. However, *P. macropilis* was able to maintain coordinated movement at higher temperatures than *P. persimilis*, suggesting that it would also be an effective control agent of *T. urticae*.

Hymenopteran species with a lower SVR appear to have a more protracted resistance of the deleterious effects of increasing temperatures compared to those with higher SVR (Le Lann *et al.*, 2011). Water loss through the cuticle will be more gradual in a species with a smaller surface area, which may delay the deleterious effects of desiccation (Renault *et al.*, 2005). This may account for the discrepancy between the upper thermal activity thresholds of the mites, as *T. urticae* is slightly larger than the two phytoseiids.

A pertinent consideration in the comparison of the phytoseiid species is the length of time for which the predators have been in culture. Both species have a common short term rearing history, with two generations at 25°C 18:6LD, although the long term rearing histories of *P. macropilis* and *P. persimilis* differ. *Phytoseiulus persimilis* has been used in biological control since 1968, but production cultures are usually refreshed with wild stock at regular intervals (van Lenteren and Woets, 1988). The mites used in these experiments were representative of those supplied commercially. Thus, although *P. macropilis* has been in commercial production for a shorter period of time than *P. persimilis*, the regular refreshing of the production system may overcome some of the issues associated with inbreeding and reduced thermal stress tolerance (Dahlgaard *et al*., 1995; Dahlgaard and Loeschcke, 1997; Dahlgaard and Hoffman, 2000; Sime *et al.*, 2006; Kristensen *et al.*, 2011).

The walking speed of *P. persimilis* was much faster than *P. macropilis*, which did not significantly differ from *T. urticae* at any temperature. The result could be due to the unnatural environment in which the mites were studied. Y-tube olfactometer experiments have shown that volatiles emitted from a plant infested with *T. urticae* elicit a strong foraging response from predatory phytoseiid mites (Janssen *et al.*, 1999; Shimoda, 2010), but in this experiment there were no volatiles or prey present to elicit foraging behaviour. An 'unnatural' surface, such as the arena, could also influence the walking behaviour of mites. In the absence of prey, *Typhlodromus pyri* Scheuten demonstrated approximately the same activity level on a bare tile as on a leaf surface, whereas *Metaseiulus* (*Typhlodromus* or *Galendromus*) *occidentalis* Nesbitt (Acari: Phytoseiidae) showed higher activity levels on leaves (Croft and Zhang, 1994). The presence of prey also altered the results; both *T. pyri* and *M. occidentalis* were less active on a leaf compared to the tile surface (Croft and Zhang, 1994). Despite their

apparent low activity levels, both species are successful biological control agents (Croft and MacRae, 1992; McMurtry and Croft, 1997; Marshall and Lester, 2001; Colfer *et al*., 2003).

The assumption that walking speeds measured in a bare arena are a reliable indicator of efficacy should therefore be further investigated as a study including leaf surface and available prey may yield a more definitive answer as to which species is the more adept predator. This investigation could encompass different plants that the predators are likely to be used on, as the morphology of the leaf surface will also determine the efficacy of the mite. Previous studies have found that glandular trichome density can inhibit the movement of some phytoseiid predators (Van Haren *et al.*, 1987; Krips *et al.*, 1999; Loughner *et al.*, 2010).

The data indicate that *P. macropilis* has the potential to forage at temperatures below the activity threshold of *T. urticae*, and as such would be an effective biological control agent in a temperate environment. The lower thermal thresholds of *P. macropilis* indicate that movement is restricted below 10°C. This data contributes to the safety assessment of *P. macropilis* as an augmentative agent, as dispersal of the mite outside a glasshouse will be constrained by northern European winters, limiting its potential to establish in the external environment. Further studies could investigate the predation behaviour of the mite on the leaf surface, including factors such as temperature and trichome density, to fully characterise the efficacy of *P. macropilis*.

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CHAPTER 5

Comparative efficacy of the spider mite predators *Phytoseiulus macropilis* and *Phytoseiulus persimilis*

5.1. Abstract

In order to characterise the predatory efficacy of *Phytoseiulus macropilis* Banks, predation of *Tetranychus urticae* Koch (Acari: Tetranychidae) was observed on leaf disk bioassays and on plant cuttings, and compared with another predatory mite, *Phytoseiulus persimilis* Athias-Henriot (Acari: Phytoseiidae). At six temperature intervals between 15 and 30°C on French bean and tomato disk bioassays, three measures of predatory efficacy were identified: time to first predation, frequency of contact with prey, and total handling time. Additionally, predator and prey mortality and time to cross the stem were measured on French bean and tomato cuttings at 20 and 25°C. There were no significant differences between *P. macropilis* and *P. persimilis* in time to first predation, frequency of contact or total handling time on either plant species at temperature intervals between 17.5 and 30°C. *Phytoseiulus macropilis* demonstrated higher levels of foraging and predation at 15°C. In the plant cutting experiments, prey mortality was higher at 25°C. Both predators displayed higher levels of mortality on tomato cuttings compared with French bean. These results indicate that the efficacy of *P. macropilis* is similar to that of *P. persimilis*, and therefore has the potential to be used as a glasshouse biocontrol agent in northern Europe.

5.2. Introduction

Whilst under attack from herbivores, plants have several defensive options: morphological adaptation, production of toxins and anti-feedants, and indirect defence through the attraction of predators. Herbivore-induced plant volatiles (HIPVs) are an indirect defence against colonisation of phytophagous species, whereby different infochemicals are released by the plant to indicate the presence of adults or eggs to natural predators (Dicke and Baldwin, 2010; Fatouros *et al.*, 2008; Kessler and Baldwin, 2001). Tritrophic systems are mutually beneficial for both plant and predator, and are often exploited by commercial agricultural growers using biological control.

Fluctuating temperature will affect the predatory behaviour of natural enemies (Ding-Xu *et al.*, 2007). Temperatures above 25°C adversely affect the movement of *Phytoseiulus persimilis* Athias-Henriot (Acari: Phytoseiidae) on the leaf surface, which interrupts the efficiency of *T. urticae* control (Skirvin and Fenlon, 2003a). Study of the thermal thresholds of a species indicates the lowest and highest temperatures at which foraging is possible, but does not fully account for the predation ability of the natural enemy on plant surfaces under different temperature regimes (Coombs and Bale, 2013). Prey handling time has been identified as a more practical gauge of predator efficiency at varying temperature intervals (Ali *et al.*, 2011).

The presence of trichomes can have a deleterious effect on populations of natural enemies. Of the seven types of trichome identified by Luckwill (1943), types I, IV, VI and VII are glandular. Glandular trichomes consist of a stalk with a collection of cells at the apex that contain several insecticidal exudates (Lin *et al.*, 1987). Upon contact with an arthropod, these

cells rupture and combine, polymerising and permanently capturing the individual, leading to mortality (Kowalski *et al*., 1992; Kennedy, 2003). This plant defence is indiscriminate, and will trap predators as well as herbivores. Glandular trichomes are indirectly injurious to overall plant defence through their cost to natural enemy fitness (Price *et al.*, 1980; Gassman and Hare, 2005).

Tomato, *Solanum lycopersicum* (Solanaceae), is a common target of *T. urticae* and is subject to biological control regimes using predatory mites. This species is known to have an abundance of type I and VI glandular trichomes, and a sparse covering of type VII (Simmons and Gurr, 2005). The glandular trichomes are located on the tomato vein axils and stems, and are deleterious to the most widely used predators of *T. urticae*: *P. persimilis* and *Neoseiulus californicus* McGregor (Acari: Phytoseiidae) (Walter, 1996; Cédola *et al.*, 2001). In restricting movement of the mites, the presence of glandular trichomes will reduce the efficacy of the predator (van Haren *et al.*, 1987). Control of *T. urticae* by *P. persimilis* is more efficient where the predators have the same initial distribution as the herbivore, and do not have to cross plant stems (Alatawi *et al.*, 2011). Therefore, it is important to account for any deleterious effects of the host plant on a potential biological control agent, and ensure the candidate is able to move within and between plants, prior to release in order to establish and utilise the most effective agent.

This study was designed to compare the predation behaviour of the spider mite predators *Phytoseiulus macropilis* Banks and *Phytoseiulus persimilis* Athias-Henriot (Acari: Phytoseiidae) on the leaf surface, and movement across the stem. *Phytoseiulus macropilis* is found in the tropics and the Mediterranean, and is a predator of *T. urticae* in glasshouse

conditions (de Moraes *et al*, 2004; Oliveira *et al*., 2007). The species is currently under investigation for suitability of use in northern European glasshouses. The data will indicate whether the efficacy of *P. macropilis* is comparable to *P. persimilis*, which has been used to control *T. urticae* since the 1960s, in predation behaviour upon the leaf surface (Oatman *et al*., 1968). The efficacy of the two predators on high trichome density plants has previously been studied; however, as temperatures above 25°C are known to adversely affect the movement of *P. persimilis*, this study encompasses the factors of temperature on plants with and without glandular trichomes, at leaf surface and plant stem scale (Sato *et al.*, 2011; Skirvin and Fenlon, 2003a).

5.3. Materials and Methods

5.3.1. Cultures

The rearing systems of *P. macropilis*, *P. persimilis* and *T. urticae* are described in Chapter 2; however, the feeding regime of the predators varied from the described methods. *Tetranychus urticae* were brushed from infested leaves on to *P. macropilis* and *P. persimilis* rearing tiles. Each culture was allowed to complete two generations under the aforementioned conditions prior to use in experiments. Adult female phytoseiids tend to be more voracious than other life stages, and were used for all experiments (Gotoh *et al.*, 2004).

Phaseolus vulgaris and *S. lycopersicum* var. 'Gardener's Delight' were grown under 22°C, 18:6 LD. French bean plants were sown three weeks prior to use in experiments, and tomato plants eight weeks prior to use.

5.3.2. Experimental system

The experimental system developed by Hazell *et al.* (2008) has previously been used to assess the thermal activity thresholds of several phytoseiids: *Phytoseiulus longipes* Evans, *Amblyseius swirskii* Athias-Henriot, *P. macropilis* and *P. persimilis* (Allen, 2010; Coombs and Bale, 2013), as well as a variety of other invertebrate species (Hazell *et al.*, 2010; Hughes *et al.*, 2010a,b). The predatory behaviour of the mites was recorded for retrospective analysis using the methods described in section 4.3.3.

5.3.3. Predatory behaviour on the leaf surface

The predatory behaviour of 30 individuals of each species was measured at six temperatures: 15, 17.5, 20, 22.5, 25 and 30°C, on French bean and tomato leaf disk bioassays. Predators were starved for 24h prior to use in the experiment to prevent previous consumption from affecting the predation behaviour recorded.

Fresh leaf discs were used in each experiment, and cut to fit the bottom of the arena without gaps under which the mites could escape. Ten *T. urticae* nymphs were added to the arena. A single *P. macropilis* or *P. persimilis* individual was then added, and the arena was filmed for 30 min for retrospective analysis. Each of the videos were then analysed for three variables: time to first predation event, total prey handling time and frequency of contact with prey. Any interactions lasting less than 5s were not included in the analysis in effort to disregard any chance encounters.

A control was also conducted whereby an individual predator was added to the arena without any plant material; 10 *T. urticae* nymphs were then added and the arena was filmed for 30

min. This was repeated until 30 individuals of each species had been recorded at each experimental temperature.

5.3.4. Predatory behaviour and movement across the plant stem

The movement of 30 *P. macropilis* and *P. persimilis* individuals across plant stems to reach *T. urticae* was observed on both French bean and tomato cuttings at two experimental temperatures: 20 and 25°C. One experimental unit consisted of clean trifoliate leaflets placed in a 'Y' formation on a bed of saturated cotton wool in a ventilated box (12 x 18 x 6 cm). The cut stem and leaf tips were held down under moist pieces of paper to prevent leaf curl and movement of mites away from the experimental area. Ten male *T. urticae* were divided between two of the leaflets, with the distal leaflet remaining clean, and left at the experimental temperature for 24h. Male *T. urticae* were used to prevent oviposition from occurring over the experimental period, and were rarely observed to move from the leaflet they were originally placed on. Experimental units were placed in one of two controlled temperature rooms: 20 or 25°C, 18:6LD, to identify any differences in predator performance at common growing conditions in glasshouses.

Predatory mites were separated, placed at their respective experimental temperature, and starved for 24h. The predator was then placed on the clean distal leaflet. Observations were made at 0.5, 1, 2, 4, 8, 24 and 48h for successful dispersal across the stem. Predator and spider mite mortality was measured at 48h.

Control samples of spider mite and predatory mite mortality were undertaken. Forty experimental units were set up, with 20 units of French bean, twenty of tomato. Ten *T. urticae*

individuals were split equally between two of the leaflets within each unit. The samples were then divided equally between 20 and 25°C, and spider mite mortality was assessed after 72h to reflect the total experimental period. In the predator control, 20 individuals of each predatory mite were placed individually into Eppendorfs, divided equally between 20 or 25°C and starved for 24h. Ten *T. urticae* males were then added to each tube. Predator mortality was then checked after 48h to reflect experimental conditions.

5.3.5. Statistical Analysis

Predatory behaviour on the leaf surface

The data from the three variables measured were $\log_{10}$ transformed and analysed using the Ryan-Joiner test for normality. Time to first predation event, total prey handling time and frequency of contact with prey were each analysed using a three-way ANOVA for significant differences and interactions between temperature, plant surface and species. Significant differences within the data were identified using Fisher's Least Significant Difference *post-hoc* comparison tests.

Predatory behaviour and movement across the plant stem

Tetranychus urticae mortality data were not normally distributed, and were therefore analysed using the non-parametric Schierer-Ray-Hare extension of the Kruskal-Wallis test. Differences were examined between predator species and plant surfaces within 20 and 25°C.

Predator mortality and stem crossing data were analysed using binary logistic regression to elicit differences between temperature and plant surface.

5.4. Results

5.4.1. Predatory behaviour on the leaf surface

Time to first encounter

Both *P. macropilis* and *P. persimilis* had similar times to first predation encounter at each temperature interval and on each plant surface (Fig. 5.1a and b). There was no significant interaction between the plant surface, predator species and exposure temperature ($p = 0.420$; $F_{10,1080} = 1.025$). Time to first encounter did not vary between species on different plant surfaces ($p = 0.315$; $F_{2,1080} = 1.157$).

There was a significant interaction between species and exposure temperature ($p = 0.038$; $F_{5,1080} = 2.366$). At 15°C, mean time to the first prey handling event by *P. macropilis* and *P. persimilis* were 20.5 and 25.8 min respectively, which were not significantly different to their respective times at 17.5°C (21.5 and 20.9 min) ($p = 0.484$). *Phytoseiulus macropilis* was significantly faster in encountering prey at 15°C than *P. persimilis* ($p = 0.001$; $F_{1,180} = 11.923$).

On French bean surfaces the mean shortest time to first encounter for both *P. macropilis* and *P. persimilis* was at 25°C (10.8 and 15.8 mins respectively). Time to first encounter at 25°C was longer for both species on tomato (16.2 and 18.6 mins respectively) and on the control surface (20.6 and 25.8 mins respectively).

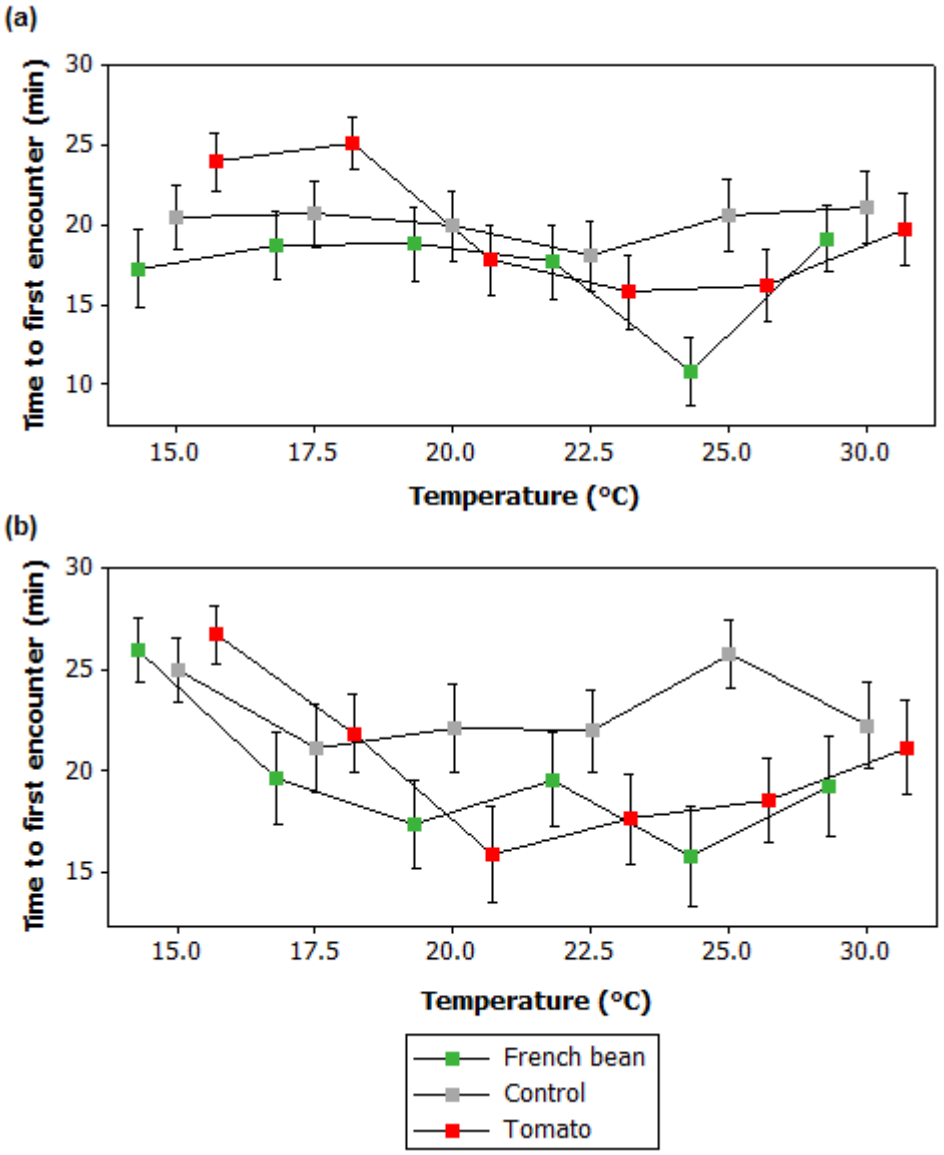

Figure 5.1. Mean (± SE) time of **(a)** *P. macropilis* and **(b)** *P. persimilis* to first encounter with *T. urticae* individuals on French bean, tomato and control surfaces at six temperature intervals

Frequency of contact

There was a low frequency of contact between each predator species and *T. urticae* across the 30 min experimental period (Fig. 5.2a and b). The frequency of contact between predator and prey significantly interacted with plant surface and temperature ($p < 0.001$; $F_{10,1080} = 6.702$).

The prominent influence on differences in the data are from the significantly higher frequency of contact between each predator and *T. urticae* on French bean at 25°C ($p < 0.001$).

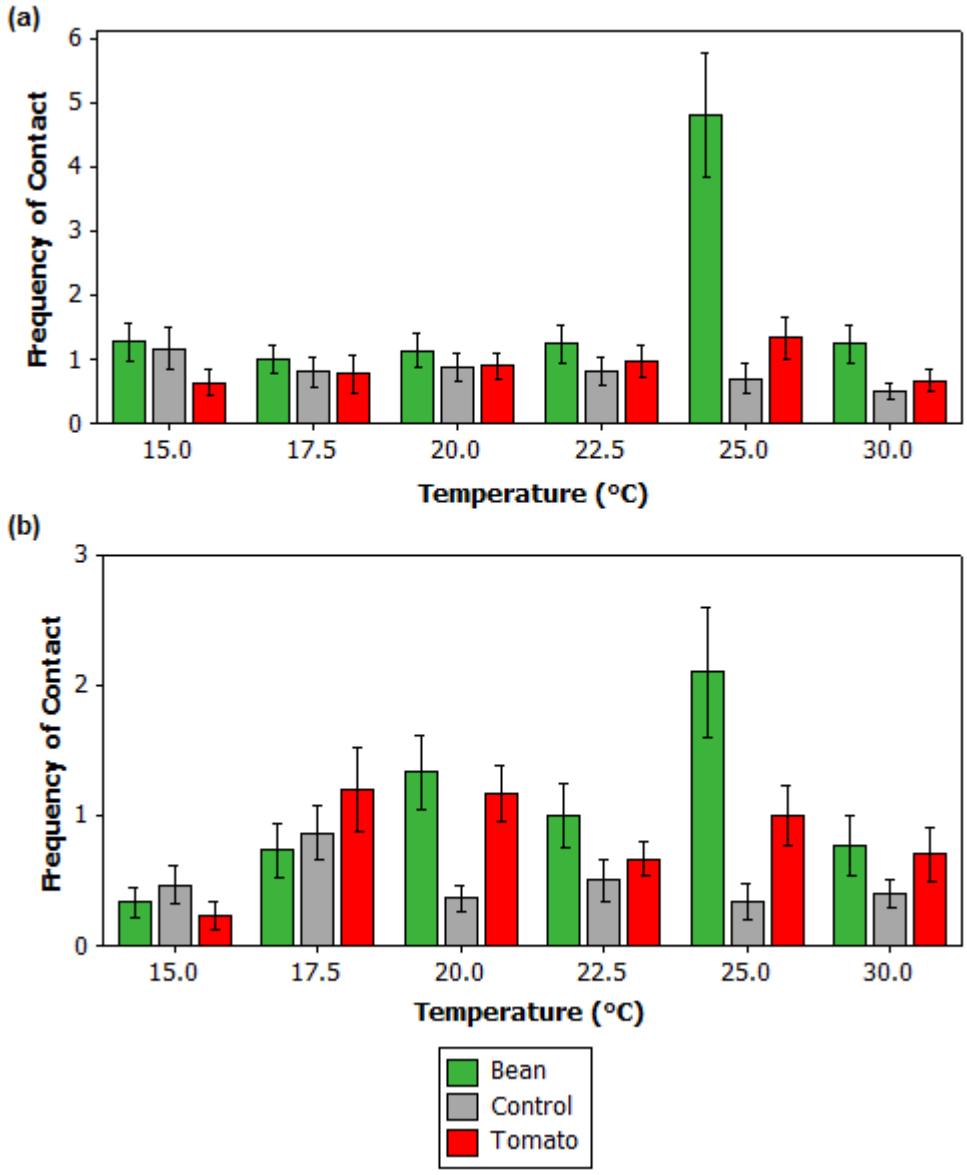

Figure 5.2. Mean (± SE) frequency of contact of **(a)** *P. macropilis* and **(b)** *P. persimilis* with *T. urticae* individuals on French bean, tomato and control surfaces at six temperature intervals.

Within this experimental condition, a one-way ANOVA identified the mean frequency of contact between *P. macropilis* and *T. urticae* as significantly higher than *P. persimilis* (4.8 and 2.1 encounters per trial respectively) ($p = 0.015$; $F_{1,59} = 6.22$). *Phytoseiulus macropilis*

had a significantly higher frequency of contact with prey compared with *P. persimilis* on each plant surface ($p = 0.044$; $F_{1,1080} = 3.137$).

Total prey handling time

At experimental intervals between 20 and 30°C *P. macropilis* and *P. persimilis* demonstrated progressively decreasing total prey handling time on tomato surfaces (Fig. 5.3).

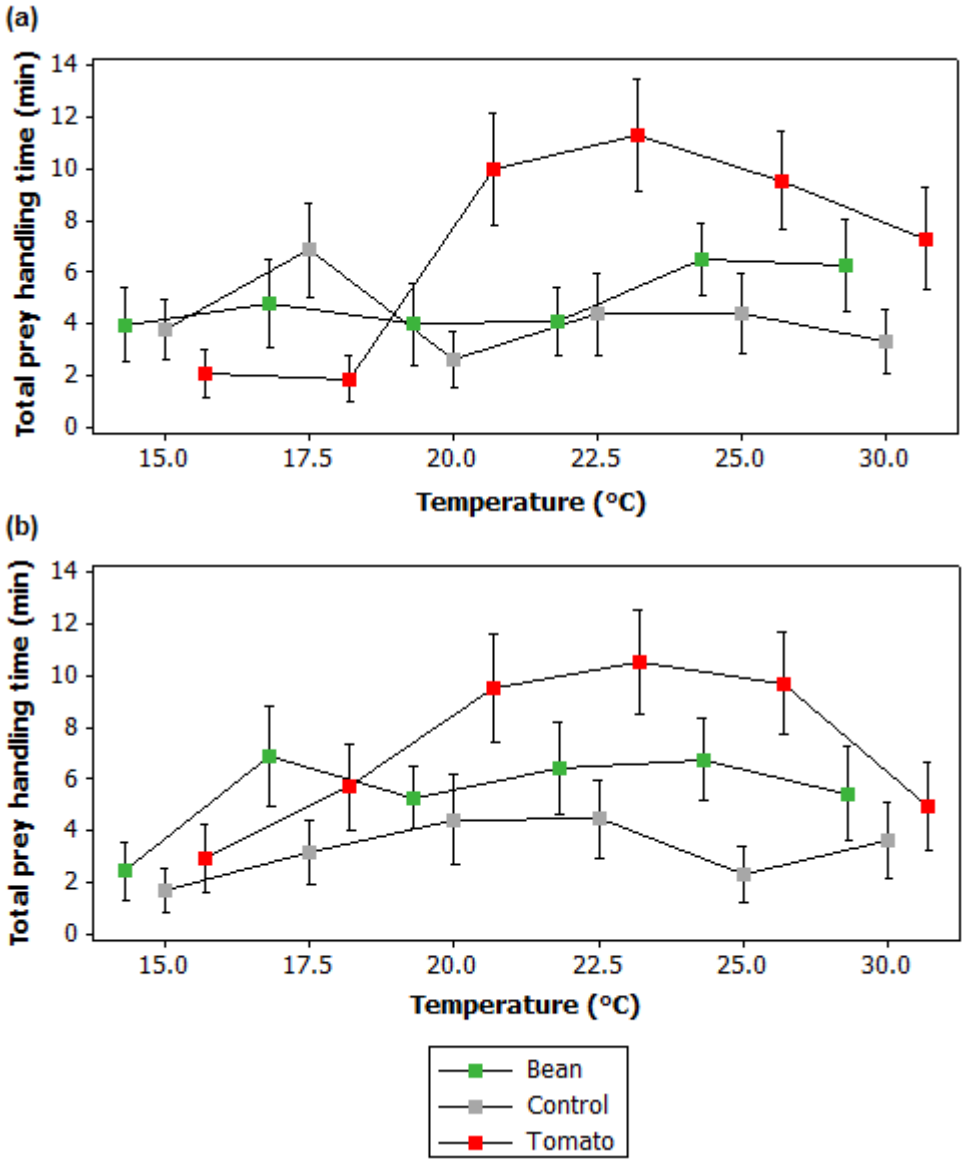

Figure 5.3. Mean (± SE) total prey handling time of **(a)** *P. macropilis* and **(b)** *P. persimilis* to first encounter with *T. urticae* individuals on French bean, tomato and control surfaces at six temperature intervals

The highest mean total prey handling times by *P. macropilis* and *P. persimilis* were at 22.5°C (11.3 and 10.5 min respectively). Prey handling time differed significantly between plant surface and temperature ($p = 0.002$; $F_{10,1080} = 2.760$). There was, however, no difference in the total prey handling time of *P. macropilis* and *P. persimilis,* neither on any plant surface nor at any temperature ($p = 0.954$; $F_{10,1080} = 0.385$).

5.4.2. Predatory behaviour and movement across the plant stem

Prey mortality

Overall, *T. urticae* mortality was significantly higher at 25°C on both French bean ($p = 0.008$; $H = 0.991$; $df = 1$) and tomato cuttings ($p = 0.01$; $H = 0.989$; $df = 1$) compared to 20°C. Within study temperatures, *T. urticae* mortality was significantly higher on French bean than tomato cuttings at both 20°C (54.7 and 23.5% respectively) ($p < 0.001$; $H = 1.000$; $df = 1$) and 25°C (66.8 and 41.7% respectively) ($p < 0.001$; $H = 0.999$; $df = 1$) (Fig. 5.4).

There was no interaction between predator species within 20°C ($p = 0.704$; $H = 0.295$; $df = 1$) or 25°C ($p = 0.203$; $H = 0.796$; $df = 1$); nor within French bean ($p = 0.91$; $H = 0.089$; $df = 1$) or tomato ($p = 0.381$; $H = 0.618$; $df = 1$). There was no *T. urticae* mortality in control conditions on French bean, and 2% mortality in the tomato control.

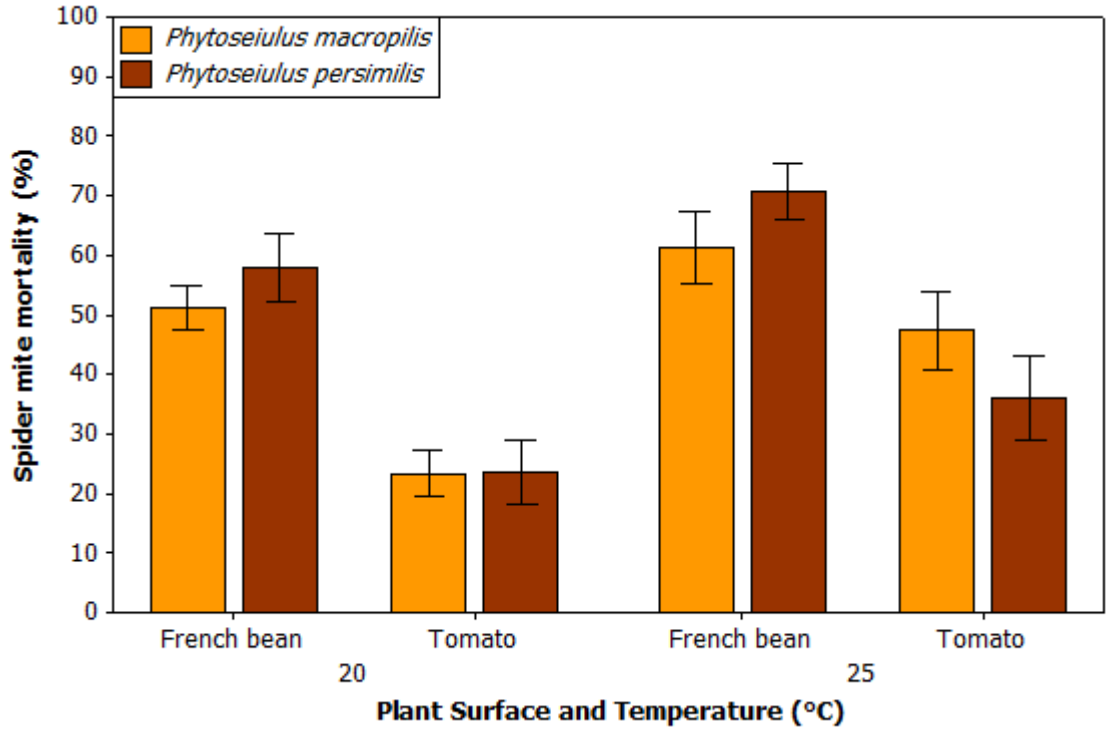

Figure 5.4. Mean (± SE) mortality of spider mites on French bean and tomato surfaces at 20°C and 25°C in the presence of either *P. macropilis* or *P. persimilis*

Predator mortality

Mortality of *P. macropilis* and *P. persimilis* were significantly higher on tomato than on French bean cuttings at both 20 and 25°C ($p = 0.036$; $Z = 2.07$; $df = 1$). Despite an apparent difference between *P. macropilis* and *P. persimilis* mortality on tomato at 25°C (20.0 and 43.3% respectively), a Kruskal-Wallis test did not elicit a significant difference ($p = 0.99$; $\chi^2 = 2.72$; $df = 1$) (Fig. 5.5). There was no mortality recorded in the predatory mite control.

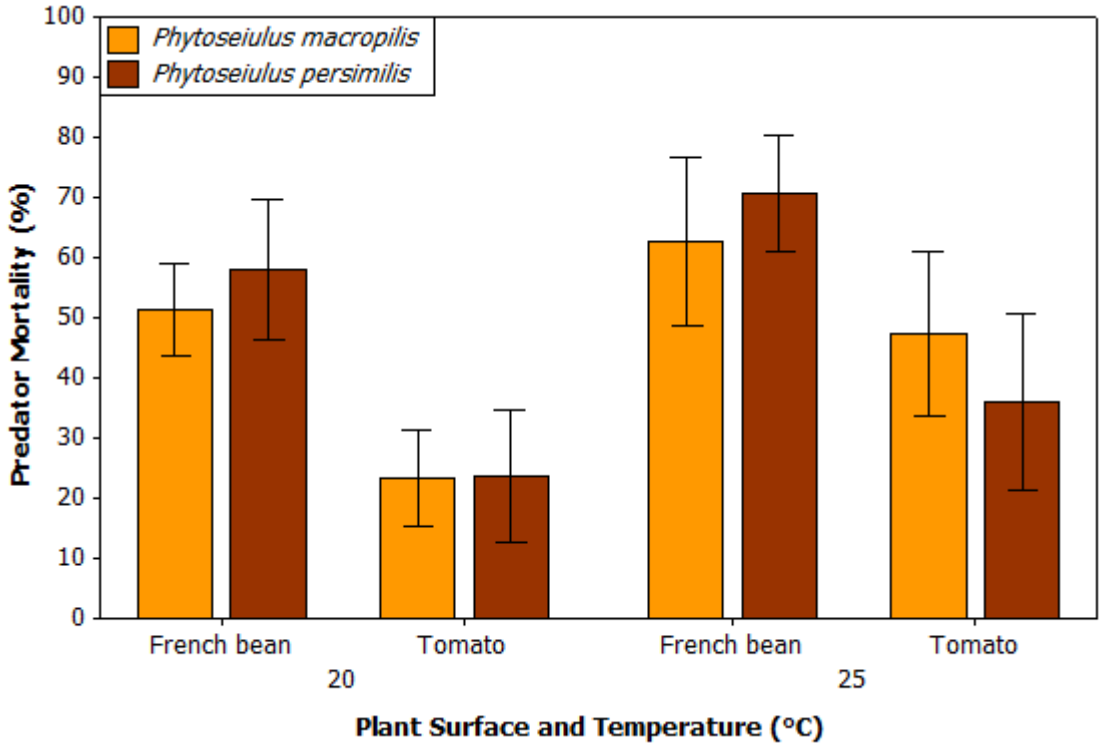

Figure 5.5. Mean (± SE) mortality of *P. macropilis* and *P. persimilis* on French bean and tomato surfaces at 20°C and 25°C

Stem crossing behaviour

There was only one experimental condition in which all individual predators crossed the stem: *P. macropilis* on French bean at 20°C (Table 5.1). Both species of predator were significantly more successful crossing French bean than tomato stems at both exposure temperatures ($p < 0.001$; $Z = 4.83$; $df = 1$); however, there was no significant difference between species on French bean ($p = 0.181$; $Z = -1.34$; $df = 1$) or tomato stems ($p = 0.741$; $Z = -0.33$; $df = 1$). Both predators were observed to take a median of 24h to cross stems of tomato cuttings at 20°C; however, *P. macropilis* was faster at 25°C compared with *P. persimilis* (0.75 and 4h respectively). In comparison, both predators were observed to take the same time to cross

French bean at 25°C (4h), but *P. persimilis* was faster at 20°C compared to *P. macropilis* (0.5 and 2h respectively).

Table 5.1. Percentage of *P. macropilis* and *P. persimilis* demonstrating successful stem crossing on French bean and tomato cuttings at 20 and 25°C

Temperature	Species	Plant	Successful stem crossing (%)
20	*P. macropilis*	French bean	100
		Tomato	63.3
	P. persimilis	French bean	90
		Tomato	50
25	*P. macropilis*	French bean	90
		Tomato	66.7
	P. persimilis	French bean	93.3
		Tomato	53.3

5.5. Discussion

In order to identify an effective biological control agent, interactions with the prey on plant surfaces at the range of temperatures likely to be experienced in a glasshouse environment should be examined. Results of predation experiments can be used to inform commercial horticultural growers in the selection of an appropriate biological control agent. Economic optimum temperatures for glasshouses in northern Europe are between 19 to 20°C, and therefore candidate biological control agents for this region, such as *P. macropilis*, should demonstrate rapid foraging behaviour and frequent predation events between these temperatures (Van Der Ploeg and Heuvelink, 2005). There were few differences between the predatory behaviour of *P. macropilis* and *P. persimilis*, a commercially available agent which is currently used in twenty countries (Cock *et al.*, 2010). The results indicate that *P. macropilis* will make an effective biological control agent in northern European glasshouses;

however the species does not exhibit a higher degree of control of *T. urticae* compared to *P. persimilis* on the plant surface, regardless of presence of glandular trichomes.

Temperature affects the level of phytoseiid activity on the leaf surface. The efficacy of several biocontrol agents, including *P. persimilis*, *Typhlodromus pyri* Scheuten and *Amblyseius cucumeris* Oudemans (Acari: Phytoseiidae) are known to decline above 25°C (Sengonca *et al.*, 2003, Skirvin and Fenlon, 2003a, Shipp *et al.*, 1996). *Neoseiulus fallacis* Garman (Acari: Phytoseiidae) was able to effectively limit *T. urticae* populations in a glasshouse between 21.1 and 26.7°C; beyond these temperatures there were greater fluctuations in predator and prey populations, and *N. fallacis* was unable to suppress the herbivore (Burnett, 1970). Prey capture rates of *Neoseiulus bakari* Hughes (Acari: Phytoseiidae), measured between 15 and 35°C, were lowest at 15°C and highest at 20°C (Jafari *et al*., 2012). *Phytoseiulus macropilis* and *P. persimilis* demonstrated diminishing performance between 25 and 30°C on both plant surfaces, as time to first encounter increased, and frequency of contact and total prey handling time decreased. Reliable indicators of species fitness have previously been identified as net reproductive rate and intrinsic rate of increase, which peak at 28 and 27°C in *P. macropilis* and *P. persimilis* respectively (Huey and Berrigan, 2001; Ali, 1998; Takafuji and Chant, 1976). The metabolism of each predator will decline above the thermal optimum due to adverse modifications to enzymes and cell membranes, resulting in a deterioration of fitness and performance (Neven, 2000). The physiological response within tomato plants are similar at 30°C, and glasshouse upper temperatures are regulated to prevent a decrease in fruit yield (Peet *et al*., 1997).There was no difference between the performances of the two predators on either plant at 30°C, suggesting *P. macropilis* is comparable with *P. persimilis,* and will therefore make an effective biocontrol agent in northern European glasshouses.

Phytoseiulus macropilis encountered significantly more prey at a faster rate than *P. persimilis* at 15°C. There was no difference between time to first encounter, frequency of encounter or handling time between *P. persimilis* and *P. macropilis* at 17.5, 20, 22.5 or 30°C on either tomato or French bean surfaces. Efficacy at lower temperatures may be beneficial in open field applications where crops are exposed to greater fluctuations in temperature; whereas glasshouse environmental temperature is regulated to meet the growth optimum of the crop (Manrique, 1993; Stanhill, 1980). At present, *P. macropilis* is only being assessed for use as a glasshouse biological control agent in northern Europe. Although glasshouse tomato crops are not exposed to temperatures below 12°C due to reduced metabolism and growth rate, improved efficacy at 15°C may influence commercial growers to augment applications of *P. persimilis* with *P. macropilis* (Criddle *et al.,* 1997). In reducing energy costs, some glasshouses are now subject to fluctuating temperature regimes with overall means of 15°C, which not only decreases developmental time and increases fecundity of predatory mites, but would be ideal for augmentative applications of *P. macropilis* (Vangansbeke *et al.*, 2013).

Handling time has been identified as a practical gauge of efficiency of a predator, as the cumulative capture and kill time can be calculated (Ali *et al.*, 2011). This measure has also been criticised in the study of efficacy of biological control agents, as low frequency of contact with the prey will supersede any benefit in a quick handling time (O'Neil, 1997; Sepúldiva and Carillo, 2008). The total prey handling time of *P. macropilis* and *P. persimilis* between 17.5 to 30°C did not significantly differ. In spite of a higher frequency of prey encounter in *P. macropilis* compared to *P. persimilis* during the leaf disk bioassay, the plant stem experiments do not show any difference between foraging capacity of the predators as

there was no difference in the number of kills made in 48h. As *P. macropilis* and *P. persimilis* are spider mite specialist predators, they demonstrate type II functional responses to increasing prey density, whereby the number of prey killed decelerates to a plateau over time (Holling, 1959; Skirvin and Fenlon, 2003b; Ferla *et al.*, 2011). Both predators demonstrated a similar predatory ability to another phytoseiid at the same prey density; at 25°C *Neoseiulus californicus* McGregor (Acari: Phytoseiidae) demonstrated a mean consumption rate of 5 adult male *Tetranychus cinnabarinus* Boisduval (Acari: Tetranychidae) per 24 h with a prey density of 10 individuals (Kuştutan and Çakmak, 2009). *Tetranychus urticae* mortality was higher after 48 h on both plants at 25°C compared to 20°C, indicating that temperature acts as a limiting factor on the predatory response of both predators.

Experiments conducted on stem crossing ability corroborated previous work on the predatory ability of *P. persimilis*. In a comparison of rearing regimes at 20, 25 and 30°C, *P. persimilis* demonstrated a higher daily consumption of *T. cinnabarinus* individuals at 25°C, and its population growth was double that of the rate at 20°C (McClanahan, 1968; Kazak, 2008). Both *P. macropilis* and *P. persimilis* were more effective at 25°C, suggesting they will exert greater control over *T. urticae* populations in glasshouses maintained at this temperature.

Both predators displayed significantly higher mortality and lower stem crossing success on tomato cuttings compared to French bean or controls. This is probably due to the growth of trichomes, which vary in resistance properties between plant species. Although glandular trichomes of strawberry plants did not affect the prey consumption rate of *N. californicus* (Gugole Ottaviano *et al.*, 2013), glandular trichomes of tomato plants are known to be deleterious to several phytoseiid agents (Walter, 1996; Cédola *et al.*, 2001; Koller *et al.*,

2007). Abundance of trichome type differ between tomato cultivars, and *S. lycopersicum* is noted for its profusion of type VI glandular trichomes. Type VI trichomes are abundant on vein axils, and are a defensive structure that indiscriminately ensnare small arthropods (Kennedy, 2003). Some predators became entrapped on the tomato leaf surface and were not observed to move during the 48 h period of the experiment, whereas there was no cessation of predator ambulation on French bean (pers. obs.). This drawback may be overcome by commercial biological control companies, as rearing history of the arthropod may influence performance on a novel plant host (Gorur *et al*., 2005). Despite a long rearing history of 300 generations on cucumber plants, *T. urticae* demonstrated phenotypically plastic responses to novel hosts, including tomato, and was able to adapt to the plant surface within 15 generations (Magalhães *et al*., 2007). If natal plant hosts are factored into commercial rearing regimes, the efficacy of both predators may be improved on tomato plants.

The webbing of *Tetranychus urticae* can inhibit the movement of predatory mites across the plant surface, or prompt foraging behaviour (Sabelis and Bakker, 1992; Kishimoto and Adachi, 2008). Both *P. macropilis* and *P. persimilis* prefer spending time on leaves with spider mite webbing compared to clean leaves of the same plant species (Roda *et al.*, 2001; Sato *et al*., 2011). Spider mite webbing was not formed during either the arena or stem experiments, the absence of which may have impeded the foraging and predatory success of *P. macropilis* and *P. persimilis*.

The information presented aims to clarify the most suitable temperature regimes required by *P. macropilis* to exert quick and effective control over an outbreak of *T. urticae* in commercial horticulture. There were no differences between the predatory behaviour of each

phytoseiid predator at exposure temperatures between 17.5 and 30°C, with an increased capability of *P. macropilis* to predate *T. urticae* at 15°C. As *P. persimilis* is a commonly used biocontrol agent of *T. urticae*, augmentation using *P. macropilis* will aid pest control in horticultural regimes exposed to lower temperatures. *Phytoseiulus macropilis*, like *P. persimilis*, is most effective at 25°C and should be applied to glasshouses where this temperature can be maintained. The data indicate that predation ability of *P. macropilis* is comparable to *P. persimilis*, and will make an effective biological control agent in climate-controlled glasshouses in northern Europe.

CHAPTER 6

Thermal biology of the spider mite predator *Balaustium hernandezi*

6.1. Abstract

Balaustium hernandezi Von Heyden (Acari: Erythraeidae) is a recently described generalist predator of several arthropod pests, including the two-spotted spider mite *Tetranychus urticae* Koch (Acari: Tetranychidae). The predator has been proposed as a candidate augmentative biological control agent in glasshouses in northern Europe. This study investigates the risk of establishment in northern Europe posed by escapees from a glasshouse environment, through the combination of laboratory and field experiments. Adult mites froze at a significantly higher temperature than larvae, but displayed low pre-freeze mortality and prolonged survival at 5°C. In contrast, the larvae had a higher level of pre-freeze mortality, but also demonstrated extended survival at 0 and -5°C. Both cohorts survived for a maximum of 112d during winter field exposures. The results indicate that *B. hernandezi* has a relatively high potential to establish in northern Europe.

6.2. Introduction

Glasshouses offer a stable environment with controlled day lengths, irradiance, irrigation and temperature, allowing crop growth and yield to be controlled and manipulated by commercial growers (Manrique, 1993; Stanhill, 1980). The climate is controlled to promote maximum crop yield, which often corresponds with the optimal environment for crop pest population growth. Historically, crop pests have been treated with chemical pesticides; however, growing

concerns about the environment and human health have created a need for alternative methods of pest control (Pimentel, 2005).

Between 1945 and 2000, insect damage to corn crop systems in the US increased from 7 to 13%, despite a tenfold increase in the use of pesticides (Pimentel *et al*., 1992). Resistance to pesticides is a recurring issue in the control of crop pests, and occurs with frequent applications due to the strong selection pressure for resistant genotypes in populations with a quick turnover (Georghiou, 1986). *Tetranychus urticae* Koch (Acari: Tetranychidae), or two-spotted spider mite, is a phytophagous pest of commercially grown flowers, vegetable and fruits, that has traditionally been controlled by pesticide application (Driestadt *et al*., 2004). The application of numerous insecticides to single plots can lead to a reduction in natural enemies and competing phytophagous pest populations, causing outbreaks of *T. urticae* (Dutcher, 2007). Where the species inhabits perennial ornamental plants there is a marked resistance to numerous acaricides due to continuous application, whereas populations found on edible crops were sprayed less due to consumer health concerns and had a much lower incidence of resistance (Stavrinides and Hadjistylli, 2009).

The use of natural enemies to control pest populations is now advancing, as it has many environmental and economic advantages over chemical control (Cory and Myers, 2000; Denoth *et al.*, 2002; Bale *et al.*, 2008b; van Lenteren, 2012). Augmentative biological control is the application of an exotic natural enemy in areas where abiotic factors prevent prolonged survival and reproduction (van Lenteren and Bueno, 2003). In Europe, non-native augmentative biological control agents are applied within the contained environment of a glasshouse, and have been used successfully to control pests such as *T. urticae* and

Trialeurodes vaporariorum Westwood (Homoptera: Aleyrodidae) (Port and Scopes, 1981; van Lenteren *et al*., 1996). Biological control is not without risk, and many EU countries regulate the use of non-native invertebrate agents (Loomans, 2007).

Predators and parasitoids reared en-masse by commercial biological control companies in Europe have to adhere to the license requirements of the country of release; for example, the UK license requires an environmental risk assessment (ERA) that has to address establishment potential, host range and dispersal ability. Conducting an ERA on a candidate augmentative biological control agent will reduce the chance of introducing a species that has the potential to become invasive. The agent should lack cold hardiness that would otherwise allow escapees from the glasshouse environment to survive a typical winter (van Lenteren *et al*., 2003; van Lenteren *et al.*, 2006; Hatherly *et al.*, 2005a). This is especially pertinent in northern Europe, where typical winter temperatures may act as a limiting factor on the growth or survival of a poikilothermic population, and thus prevent a species from establishing and possibly becoming invasive.

This study is designed to investigate the survival and establishment potential of *Balaustium hernandezi* von Heyden (Acari: Erythraidae) in northern Europe. *Balaustium hernandezi* is a recently described species, originating from the Mediterranean, and intended for use in controlling phytophagous mites such as *T. urticae* (Mąkol *et al.*, 2012). Although little is known about this particular species' thermal hardiness, the genus is known for its remarkable high temperature tolerances (Yoder *et al*., 2007a; Yoder *et al*., 2008; Hedges *et al.*, 2012). The genus is known to be polyphagous, targeting moths, butterflies, scales and other mites as prey (Putman, 1970; Cadogan and Laing, 1977; Hayes, 1985). Some members of the genus

also utilise pollen and leaf tissues as food resources, and have been recorded to bite humans, though this behaviour is not ubiquitous within the genus (Newell, 1963). In this study, *B. hernandezi* was studied for its potential to establish outside of a glasshouse environment over a typical northern European winter. The data were intended to indicate whether *B. hernandezi* is safe for use in glasshouses of those northern European countries that require an ERA to be completed as part of the requirements of invertebrate biological control regulation.

6.3. Materials and Methods

6.3.1. Cultures and experimental procedures

Rearing methods of the mites are described in Chapter 2. Supercooling, lethal temperature, lethal time and field trial experiments have previously been used to assess the cold hardiness of several candidate biological control agents (Allen, 2010; Hughes *et al.*, 2009, 2011).

6.3.2. Supercooling point

The supercooling points (SCP) of adult and larval *B. hernandezi* were assessed using the methodology described in Bale *et al*. (1984). Supercooling points were measured with a Pico TC-08 Thermocouple Data Logger. Individual mites were attached to type K thermocouples using a small volume of Oecotak and then placed in size three Beem capsules (Ladd Research, USA). The capsules were placed in boiling tubes suspended in a programmable alcohol bath. The temperature was reduced at 0.5°C min^{-1} from 25 to -30°C, and the supercooling point recorded as the temperature at which an exotherm was detected.

6.3.3. Lethal temperatures

Lethal temperature (LTemp) experiments determined the temperatures at which each cohort showed 10, 50 and 90% mortality. Individual *B. hernandezi* were placed into size three Beem capsules. Five capsules were loaded into a boiling tube, with 6 boiling tubes per treatment, and placed into a programmable alcohol bath. The temperature was then either increased or decreased from 25°C at 0.5°C min^{-1} to each exposure temperature, held for 10 min, and then returned to 25°C at the same ramping rate. Treated individuals were then placed separately into test eppendorfs and held in rearing conditions. Mortality was recorded 72h after the exposure.

In the lower LTemp experiments a range of exposure temperatures between 2 and -10°C were investigated, and in the upper LTemp experiments the exposure temperatures were between 34 and 50°C. Both ranges were predetermined by preliminary experiments that indicated the range over which 0 and 100% mortality occurred.

6.3.4. Lower lethal times

The lower lethal time (LTime) experiments calculated the time at which each cohort exhibited 10, 50 and 90% mortality at 5, 0 and -5°C. These conditions were chosen to represent typical mild, moderate and more severe northern European mean winter temperatures respectively. *Balaustium hernandezi* adults and larvae were individually placed into test Eppendorfs. A total of 210 test Eppendorfs per cohort were placed at each exposure temperature, allowing samples of three replicates of ten mites of each cohort. All Eppendorfs were held at 10°C for 1h to counter cold shock prior to immediately placing at 5, 0 or -5°C.

Samples were removed at different time intervals determined by preliminary experiments, and held at 10°C for 1h to counter heat shock. Individuals were then placed in a test Eppendorf and returned to rearing conditions. Mortality was assessed 72h after the exposure.

6.3.5. Field trials

Individual adult and larval *B. hernandezi* were transferred into test Eppendorfs, and exposed to winter field conditions. Test Eppendorfs were placed in sealed ventilated boxes, and transferred to 10°C for 1h to counter mortality from cold shock. The boxes were then placed in a sheltered location on the campus of the University of Birmingham. Two Tinytag ® dataloggers were placed in each box to record the temperature at 5 min intervals throughout the trial. A fresh supply of *T. urticae* was transferred to the test Eppendorfs every 5 days. Samples of 30 test Eppendorfs per cohort were removed from the field and transferred to 10°C for 1h to prevent mortality from heat shock. *Tetranychus urticae* nymphs were added to the Eppendorfs, placed into rearing conditions and mortality was assessed after 72h.

Winter 2011

Balaustium hernandezi were exposed in the field from 23rd November 2011 to 30th March 2012. Adults and larvae were sampled at 1, 3, 7, 14, 28, 56 and 112 days.

Winter 2012

Adult and larval *B. hernandezi* were exposed between 30th October and 28th December 2012, and sampled at 7, 22, 28, 36, 42 and 59 days.

6.3.6. Control samples

Thirty mites were placed individually into size three Beem capsules, which were distributed between six boiling tubes and placed into the alcohol bath. The mites were held at 25°C for the maximum experimental period (2h 30m). Individuals were then placed into test Eppendorfs and held in rearing conditions. Mortality was recorded 72h after the exposure.

A combined control was run for the lower lethal time and field trial as both experiments required maintaining *B. hernandezi* in test Eppendorfs for long periods of time. As the average lifespan of adult *B. hernandezi* in rearing conditions was 21d, a control population maintained under these conditions would not survive periods mirroring the lethal time and field trial experimental periods (72 and 112 days respectively). Thus, 30 individuals were placed into test Eppendorfs and held for 14d in rearing conditions. Mortality was recorded 72h after the exposure.

6.3.7. Statistical Analysis

All statistical analyses were made using Minitab 16 (Minitab Ltd., Coventry, UK). After testing for normality and equal variances, differences between the supercooling points of adults and larvae were determined using an ANOVA. The results of lower lethal temperature and time experiments were analysed using SPSS 21 (IBM). Data were tested for normality using Pearson's Goodness-of-Fit test, and analysed using probit analysis (Finney 1971). Temperatures and times resulting in 10, 50 and 90% mortality were selected from the output, and any differences between adult and larvae identified through a Parallelism Test, which tests whether each factor level has a common slope. Field trial data were analysed using

binary logistic regression to elicit differences between adults and larvae across the experimental period. Results were considered significant when $p < 0.05$.

6.4. Results

6.4.1. Supercooling point

Adult *B. hernandezi* had a mean SCP of -7.4 ± 0.2°C, a significantly higher mean SCP than larvae (-18.4 ± 0.6°C) ($p < 0.001$; $F_{1,58} = 311.96$).

6.4.2. Lethal temperatures

The probit estimates and 95% confidence intervals for lower and upper lethal temperature limits are displayed in Table 6.1. There was no mortality recorded in the control sample.

Table 6.1 Parameter estimates of Probit analysis of upper and lower lethal temperatures

	Parameter	Cohort	Estimate	Std. Error	Z	Sig.	95% Confidence Interval	
							Lower bound	Upper bound
Upper lethal temperature[a]	Temperature		35.816	3.550	10.089	< 0.001	28.858	42.774
	Intercept	Adult	-59.337	5.873	-10.104	< 0.001	-65.209	-53.464
		Larvae	-57.273	5.728	-9.999	< 0.001	-63.001	-51.546
Lower lethal temperature[b]	Temperature		-3.093	0.407	-7.596	< 0.001	-3.890	-2.295
	Intercept	Adult	1.825	0.331	5.511	< 0.001	1.494	2.157
		Larvae	2.654	0.361	7.357	< 0.001	2.293	3.014

[a]PROBIT model: PROBIT(p) = Intercept + BX (Covariates X are transformed using the base 10.000 logarithm.)
[b]PROBIT model: (PROBIT(p) + 11) = Intercept + BX (Covariates X are transformed using the base 10.000 logarithm.)

The upper lethal temperature data were not normally distributed ($p < 0.001$; $\chi^2 = 36.809$; $df = 8$), and therefore a heterogeneity factor was used in the calculation of confidence intervals, allowing for the increased variance within the results (Lacey, 1997). The upper lethal temperature$_{10,50,90}$ of adults was higher than of the larvae, and the Parallelism Test determined a significant difference between cohorts ($p < 0.001$; $\chi^2 = 34.731$; $df = 1$) (Table 6.2).

Table 6.2 Estimated lethal temperatures resulting in 10, 50 and 90% mortality in adult and larval *B. hernandezi*

	Cohort	Mortality (%)	Estimate (°C)	Std. Error	95% Confidence Interval	
					Lower bound	Upper bound
Upper lethal temperature[a]	Adult	10	41.775	1.012	37.983	43.800
		50	45.363	1.010	43.152	47.894
		90	49.258	1.014	46.888	54.755
	Larvae	10	36.586	1.015	32.303	38.548
		50	39.728	1.009	37.304	41.467
		90	43.140	1.011	41.338	46.485
Lower lethal temperature	Adult	10	-0.891	1.123	-2.709	2.360
		50	-7.107	1.108	-7.930	-6.330
		90	-9.501	1.219	-10.098	-8.942
	Larvae	10	7.728	1.166	3.592	16.673
		50	-3.788	1.086	-4.883	-2.456
		90	-8.222	1.158	-9.081	-7.474

[a]A heterogeneity factor is used as Pearson $\chi^2 > 0.15$

The lower lethal temperature data were normally distributed ($p = 0.189$; $\chi^2 = 9.998$; $df = 7$). The lower lethal temperature$_{10,50,90}$ of adults were higher than of the larvae, however, the result of the Parallelism Test indicated that there was no significant difference between adults and larvae ($p = 0.682$; $\chi^2 = 0.167$; $df = 1$). The lower lethal temperature$_{50}$ was higher than the SCP for both cohorts.

6.4.3. Lower lethal times

The probit estimates and 95% confidence intervals for lethal time at 5, 0 and -5°C are shown in Table 6.3. The data were normally distributed in the 5°C ($p = 0.598$; $\chi^2 = 6.439$; $df = 8$) and -5°C ($p = 0.539$; $\chi^2 = 6.977$; $df = 8$) treatments, but not in the 0°C treatment ($p = 0.002$; $\chi^2 = 25.841$; $df = 9$). A heterogeneity factor was used in the calculation of confidence intervals for adults and larvae exposed to 0°C. No mortality was recorded in the control treatment.

Table 6.3 Parameter estimates of Probit analysis of lethal time at 5, 0 and -5°C

	Parameter	**Cohort**	**Estimate**	**Std. Error**	**Z**	**Sig.**	**95% Confidence Interval**	
							Lower bound	**Upper bound**
Lethal time at 5°C[a]	Temperature		2.918	0.319	9.138	< 0.001	2.292	3.544
	Intercept	Adult	-4.648	0.529	-8.786	< 0.001	-5.177	-4.119
		Larvae	-2.230	0.292	-7.641	< 0.001	-2.522	-1.938
Lethal time at 0°C[a]	Temperature		2.671	0.280	9.548	< 0.001	2.123	3.219
	Intercept	Adult	-5.539	0.576	-9.624	< 0.001	-6.115	-4.963
		Larvae	-4.322	0.504	-8.579	< 0.001	-4.826	-3.818
Lethal time at -5°C[a]	Temperature		0.620	0.150	4.117	< 0.001	0.325	0.915
	Intercept	Adult	1.036	0.186	5.576	< 0.001	0.850	1.222
		Larvae	-0.017	0.172	-0.099	0.921	-0.189	0.155

[a]PROBIT model: PROBIT(p) = Intercept + BX (Covariates X are transformed using the base 10.000 logarithm.)

Although adult survival was prolonged at 10, 50 and 90% mortality at 5°C (14.2, 39.2 and 107.7d respectively) compared with the larvae (2.1, 5.8 and 16.0d respectively), the parallelism test elicited no significant difference between the cohorts ($p = 0.742$; $\chi^2 = 0.108$; $df = 1$). This was also true at 0°C ($p = 0.192$; $\chi^2 = 1.699$; $df = 1$). Larvae demonstrated an extended lethal $time_{50}$ at -5°C compared to adults (1.06 and 0.02h respectively); the difference between the cohorts was significantly exaggerated at the lethal $time_{90}$ (2.5 and 124.7h

respectively). Despite the rift between these results, the parallelism test elicited no difference between cohorts (p = 0.240; χ^2 = 1.378; *df* = 1). The observed 100% mortality points in each lethal time investigation for adults at 5 and 0°C, and larvae at -5°C, were at 85 d, 216 h and 72 h respectively, which were all lower than the respective lethal $time_{90}$ probit results (Table 6.4).

Table 6.4 Estimated lethal time at 5, 0 and -5°C resulting in 10, 50 and 90% mortality in adult and larval *B. hernandezi*

	Cohort	**Mortality (%)**	**Estimate**	**Std. Error**	**95% Confidence Interval**	
					Lower bound	**Upper bound**
Lethal time at 5°C (days)	Adult	10	14.250	1.159	10.041	18.234
		50	39.174	1.093	32.760	46.563
		90	107.693	1.160	85.538	148.583
	Larvae	10	2.114	1.182	1.446	2.756
		50	5.811	1.099	4.791	6.929
		90	15.976	1.143	12.825	21.567
Lethal time at 0°C (hours)[a]	Adult	10	39.257	1.102	17.822	59.018
		50	118.498	1.067	83.836	174.761
		90	357.685	1.120	227.533	896.938
	Larvae	10	13.750	1.758	4.735	23.154
		50	41.504	1.270	25.223	60.543
		90	125.279	1.378	83.387	255.092
Lethal time at -5°C (hours)	Adult	10	< 0.001	2.509	< 0.001	0.005
		50	0.021	1.604	< 0.001	0.148
		90	2.492	1.336	0.557	9.035
	Larvae	10	0.009	2.095	< 0.001	0.090
		50	1.065	1.373	.124	2.634
		90	124.745	1.447	44.511	1636.811

[a]A heterogeneity factor is used as Pearson $\chi^2 > 0.15$

6.4.5. Field trials

Winter 2011

The minimum, mean and maximum temperatures of the sampling period were -5.4, 5.4 and 16.8°C respectively. The temperatures experienced by the mites, and corresponding *B. hernandezi* mortality are shown in Fig. 6.1a and b.

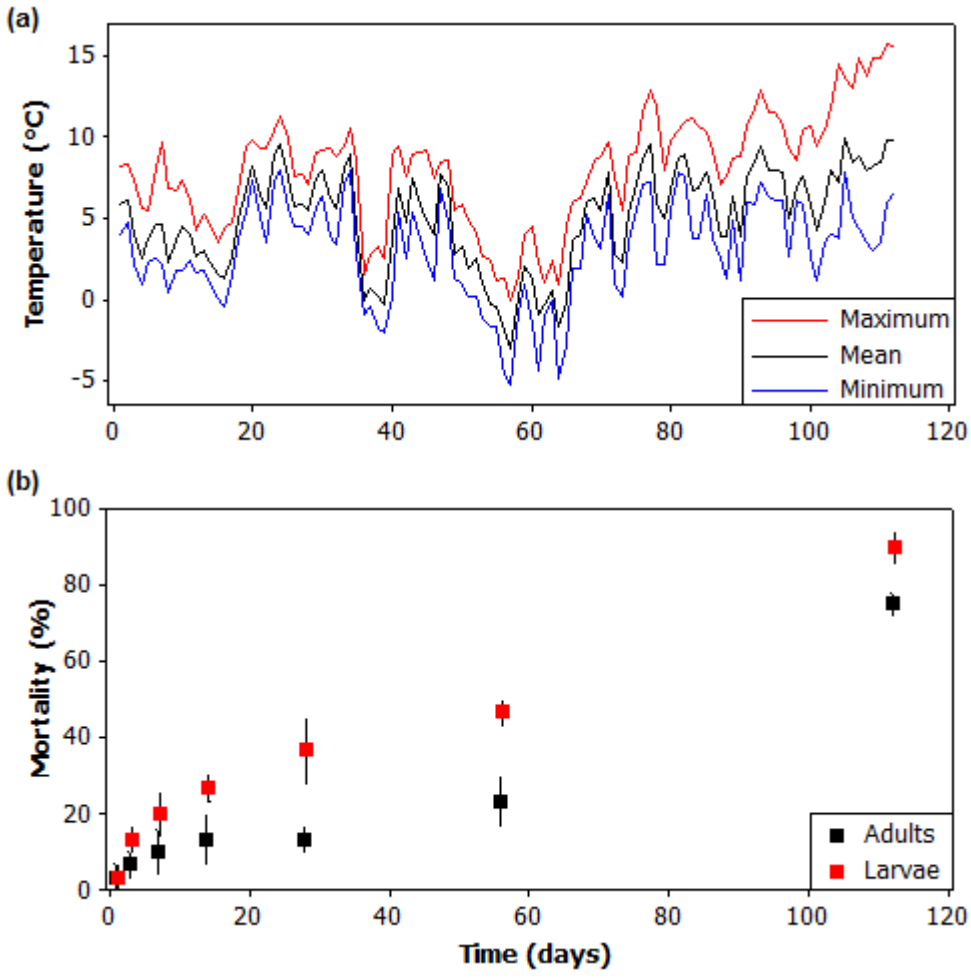

Figure 6.1. (a) Maximum, mean and minimum temperatures experienced by *B. hernandezi* in the field from 23rd November 2011 to 30th March 2012 **(b)** Mortality (mean ± SE) of adult and larval *B. hernandezi* in the field from 23rd November 2011 to 30th March 2012

Binary logistic regression was used to elicit any significant differences between the cohorts, and data were assessed for goodness-of-fit using Pearson Chi-Square statistic ($p = 0.789$; $\chi^2 =$

7.124; *df* = 11). Though neither cohort reached 100% mortality during the trial, larvae were more susceptible to the fluctuating winter temperatures than adults, and demonstrated significantly higher mortality compared to adults across the field trial period (Table 6.5). Larvae had a higher mortality rate by 112d compared with adults (90 ± 4% and 75 ± 2% respectively).

After removal from the field and 72h in rearing conditions, surviving *B. hernandezi* individuals were transferred to a rearing box and monitored for survival and reproduction. Larvae were unable to develop through to adult, and no oviposition by adults was observed.

Table 6.5 Parameter estimates of binary logistic regression of the 2011 and 2012 field exposures of adult and larval *B. hernandezi*

Year	Parameter	Estimate	Std. Error	Z	Sig.	Odds Ratio	95% Confidence Interval	
							Upper Bound	Lower Bound
2011	Constant	-3.804	0.502	-7.58	< 0.001			
	Cohort	0.982	0.271	3.62	< 0.001	1.04	1.03	1.04
	Time	0.035	0.003	10.21	< 0.001	2.67	1.57	4.54
2012	Constant	-1.458	0.555	-2.63	0.009			
	Cohort	-0.274	0.303	-0.90	0.366	0.76	0.42	1.38
	Time	0.113	0.013	8.61	< 0.001	1.12	1.09	1.15

Winter 2012

The minimum, mean and maximum temperatures experienced by the mites were -4.8, 4.9 and 12.1°C respectively. The temperatures and corresponding mortality of mites are shown in Figs. 6.2 a and b. Data were assessed for goodness-of-fit using the Pearson Chi-Square statistic (p = 0.406; χ^2 = 9.341; *df* = 9). There was no significant difference between the

mortality of adult and larval *B. hernandezi*, and both cohorts reached 100% mortality by 59d (Table 6.5).

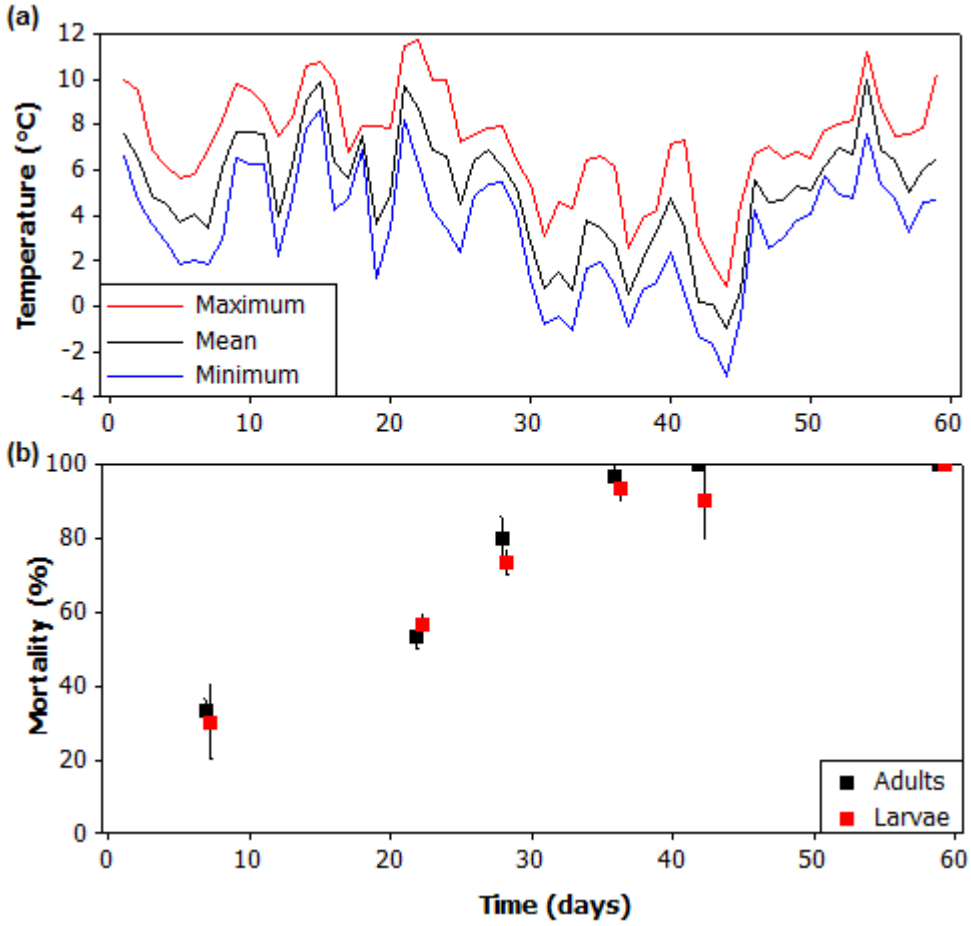

Figure 6.2. (a) Maximum, mean and minimum temperatures experienced by *B. hernandezi* in the field from 30th October to 28th December 2012 **(b)** Mortality (mean ± SE) of adult and larval *B. hernandezi* in the field from 30th October to 28th December 2012

6.5. Discussion

Ideally, a candidate augmentative biological control agent should demonstrate limited cold hardiness to reduce dispersal potential (van Lenteren *et al.*, 2003; van Lenteren *et al.*, 2006). Several candidate augmentative glasshouse biocontrol agents have been subject to a combination of laboratory experiments and field trials investigating cold tolerance, and resulting data are used to determine the risk of escapee establishment outside the glasshouse

environment in northern Europe (Hatherly *et al.*, 2005a). *Balaustium hernandezi*, an erythraeid with a Mediterranean distribution (Mąkol *et al.*, 2012), was subject to these experiments to determine the likelihood of winter survival.

There was a noticeable difference in the SCPs of adult and larval *B. hernandezi*. The relatively high SCP of adults appears consistent with a freeze tolerant species (Zachariassen, 1985; Bale 1993); however, no survival was recorded below the SCP. Mites are regarded as a freeze intolerant taxon and therefore any freezing is lethal (Sømme, 1999). In contrast, the larvae supercooled to a significantly lower temperature, which is more consistent with a chill intolerant taxon (Bale, 1993). Despite a lower SCP, larvae demonstrated increased mortality at higher sub-zero temperatures compared with adults. The adult lower $LTemp_{50}$ and $LTemp_{90}$ fell within the range of adult SCPs. The observed mortality of adults increased from 25 to 70% between -6 and -8°C, indicating that many individuals froze during these exposure temperatures. Interestingly, the adults demonstrated a significantly extended $LTime_{90}$ at 5°C compared to larvae, but at both 0 and -5°C larval $LTime_{90}$ survival was more prolonged than of adults.

In combination, the SCP, lower lethal temperature and lethal time data indicate that the adults and larvae of *B. hernandezi* utilise different cold tolerance strategies. The low SCP, relatively high $LTemp_{50}$ and low $LTime_{50}$ at 5°C denote larvae as a chill intolerant cohort. The adults demonstrated the inverse, implying that they are a strongly chill tolerant cohort (Bale, 1993). *Balaustium hernandezi* undergoes alternating calyptostasy, a developmental process whereby the prelarva, protonymph and tritonymph are functionally and morphologically regressive; all other stages are free living and active (Belozerov, 2008a; Mąkol *et al.*, 2012). A lower SCP in

larval stages is advantageous as the regressive instars will remain immobile in the soil, and thus cannot escape the deleterious effects of decreasing temperature (Terblanche *et al.*, 2007). By depressing the SCP, there is a higher chance of survival at extended periods of sub-zero temperature, which is illustrated by the increased survival of larvae at -5°C compared with adults. Although each cohort demonstrated different cold tolerance strategies, both adults and larvae exhibit the potential to endure the conditions of a northern European winter.

Winter field trial data indicate a considerable capacity to survive fluctuating and sub-zero temperatures, regardless of a distinct difference between the maximum survival times of *B. hernandezi* in the 2011 and 2012 winter field trials. The 2011 field trial did not elicit 100% mortality of either cohort during an exposure period of 112d, whereas all individuals had died by day 59 in the 2012 trial. This difference could be due to the lower mean temperature throughout the 2012 trial, despite the minimum exposure temperature being higher than the 2011 trial minimum. Decreasing temperature will slow the rate of metabolic processes and development within a poikilothermic organism to a lower threshold, below which development is not possible (Campbell *et al.*, 1974). Reactive Oxygen Species (ROS) are a byproduct of aerobic metabolism, and are produced in greater quantities during stress (Rojas and Leopold, 1996; Lalouette *et al.*, 2011). The accumulation of ROS during cold environmental conditions will cause oxidative damage to molecules within the cell, slowing the metabolism and causing injury that can lead to mortality (Monaghan *et al.*, 2009). There may have been a faster rate of accumulation of ROS in *B. hernandezi* in the 2012 trial due to the lower mean trial temperature, causing the sample population to perish within a quicker time frame than the 2011 sample population.

The combination of the 2011 *B. hernandezi* field trial data and $LTime_{50}$ at 5°C indicates that the species has an overwintering ability comparable with *Neoseiulus californicus* McGregor and *Dicyphus hesperus* Knight (Heteroptera: Miridae), which have been classed as high risk species, and were not regarded as safe for use as augmentative biological control agents in northern Europe (Hatherly *et al*., 2008). Survival of the 2012 field trial was lower, and places *B. hernandezi* in the same range as *Phytoseiulus longipes* Evans (Acari: Phytoseiidae), which is classed as presenting a 'marginal' risk of establishment in northern Europe (Allen, 2010) (Fig. 6.3). Following release as a glasshouse augmentative agent in the UK, *N. californicus* has been recorded on strawberry plants and in orchards, suggesting species in the same range pose a significant threat of establishment (Jolly, 2000; Hart *et al.*, 2002b). Data suggest any *B. hernandezi* glasshouse escapees would have the capacity to survive a northern European winter, and therefore present a significant risk of establishment. It should be noted, however, that risk of establishment is not itself an indication of subsequent negative environmental effects on native organisms, only the potential for this to occur.

Experiments in this study were conducted on the active larval and adult calyptostases. The regressive stages are protected by a thick hypodermis, cuticlin, and an epidermis; and develop underneath the detached cover of the previous instar, which is eventually shed (Shatrov, 1999; Shatrov, 2001). The insulating action of the previous calyptostases may provide the mite with some protection from the external environment, and so enhance survival of cold winter conditions. In addition, diapause has been observed in the egg stages of *Balaustium putmani* Smiley and *Balaustium murorum* Hermann (Acari: Erythraeidae), and would aid survival of the harsh conditions of winter (Putman, 1970; Wohltmann, 2001; Belozerov, 2008b). Study of the more susceptible instars demonstrates a substantial capacity to withstand a northern

European winter, and therefore indicates that *B. hernandezi* poses a significant risk of establishment.

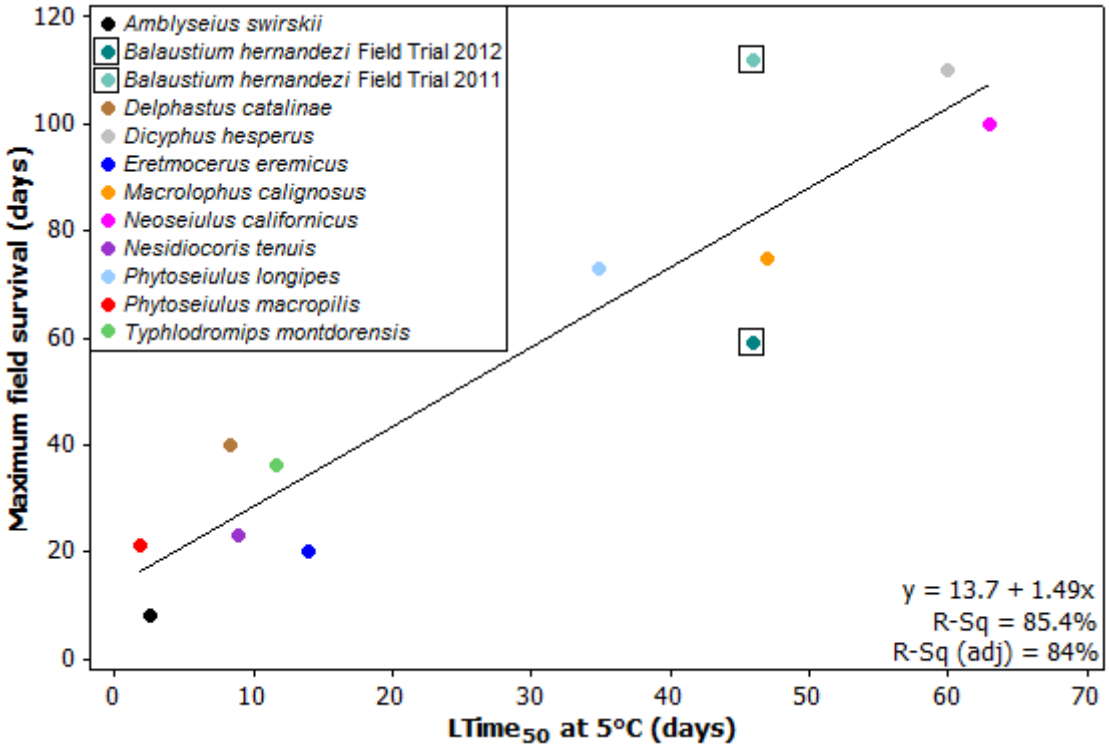

Figure 6.3. Correlation between $LTime_{50}$ at 5°C and maximum unfed field survival time of several adult biological control agents. Sources of data: *Amblyseius swirskii* (Allen, 2010); *Delphastus catalinae* (Tullett, 2002); *Dicyphus hesperus* (Hatherley *et al.*, 2008); *Eretmocerus eremicus* (Tullett *et al.*, 2004); *Macrolophus calignosus* (Hart *et al.*, 2002a); *Neoseiulus californicus* (Hart *et al.*, 2002b); *Nesidiocoris tenuis* (Hughes *et al.*, 2009); *Phytoseiulus longipes* (Allen, 2010); *Typhlodromips montdorensis* (Hatherley *et al.*, 2004).

In contrast, the upper LTemp data supports the use of *B. hernandezi* as an augmentative biocontrol agent in its native distribution in Spain. Though the internal climate is controlled, highs of 36 to 37°C have previously been recorded inside Mediterranean glasshouses (Montero *et al.*, 2001; Fargues *et al.*, 2003). As both the $LTemp_{50}$ and the temperature at which the species is unable to maintain coordinated movement are above 40°C (Coombs and

Bale, *in press*), it is unlikely that the extremes within the glasshouse environment would negatively affect the *B. hernandezi* population.

There are additional safety concerns in using this species as a glasshouse biocontrol agent outside of its native distribution. *Balasutium hernandezi* has been observed to feed on thrips and white fly, as well as spider mites (Mąkol *et al.*, 2012). Polyphagy, though desirable if the predator is targeting numerous pest species, may be problematic if the species also predates other non-target beneficial arthropods (De Clercq, 2002). *Harmonia axyridis* Pallas (Coleoptera: Coccinellidae) is a highly polyphagous species that was commercially released as biological control agent in Europe during the 1990s, but has since dispersed and by 2007, was established in at least thirteen European countries (Koch, 2003; Brown *et al.*, 2008; Raak-van den Berg *et al.*, 2012). The coccinellid presents a threat to many non-target aphidophages through competition and intraguild predation (Sato *et al.*, 2005). Other non-target organisms such as butterflies, parasitoids and syrphids are negatively affected by the presence of *H. axyridis* (Koch *et al*., 2003; Snyder *et al*., 2004; Ingels and De Clercq, 2011). The capacity to predate a wide range of non-target species, including other pests, is therefore a further factor that needs to be considered in the ERA of *B. hernandezi*.

The combination of cold tolerance data and the polyphagous nature of *B. hernandezi* suggests that the species may be unsuitable for use as a biocontrol agent in glasshouses in northern Europe. Should the mite escape from the glasshouse environment there is significant risk of establishment, and with an unknown but possible threat of predation to native invertebrate non-target species. Thermal biology of *B. hernandezi* supports augmentation within its native

distribution as it is able to tolerate the high temperatures likely to occur in Mediterranean glasshouses and open field locations.

CHAPTER 7

Thermal thresholds of the predatory mite *Balaustium hernandezi*

7.1. Abstract

The lower and upper thermal activity thresholds of adult and larval *Balaustium hernandezi* von Heyden (Acari: Erythraeidae) were compared with that of its prey *Tetranychus urticae* Koch (Acari: Tetranychidae). Adult female *B. hernandezi* retained ambulatory function (CT_{min}) and movement of appendages (chill coma) at significantly lower temperatures (5.9 and -2.1°C respectively) than that of larval *B. hernandezi* (8.1 and -1.7°C) and *T. urticae* (10.6 and 10.3°C). There was no significant difference between the temperature at which adult and larval *B. hernandezi* and *T. urticae* ceased walking as temperature was raised (CT_{max}) (46.7, 46.3 and 47.3°C respectively). Both cohorts of *B. hernandezi* ceased movement (heat coma) below the upper locomotory limits of *T. urticae* (46.8, 46.7 and 48.7°C respectively). Adult *B. hernandezi* had significantly faster walking speeds than larvae and *T. urticae* across a range of temperatures. The lower thermal activity threshold data indicate that *B. hernandezi* would make an effective biological control agent in temperate climates; however, the extent of the low temperature tolerances of the species suggests potential to establish in a northern European climate.

7.2. Introduction

Augmentative biological control is the application of an exotic natural enemy in areas where abiotic factors prevent prolonged survival and reproduction, and is considered to be an environmentally safe and cost effective method of crop pest management (van Lenteren and

Bueno, 2003; van Lenteren, 2012). Invertebrate augmentative biological control agents must, however, undergo an environmental risk assessment prior to use in many EU countries, including the UK, to prevent the introduction of a species that has the potential to become invasive. In order to comply with these guidelines, a potential biological control agent must be assessed using methodologies to determine establishment potential and host range (Wapshere, 1974, van Lenteren *et al.*, 2003; Hatherley *et al.*, 2005a; van Lenteren and Loomans, 2006). Augmentation is the prevalent form of biological control in Europe, and is predominantly used in glasshouse horticulture. Previous investigations have concentrated on low winter temperatures preventing the establishment of non-native natural enemies that escape from glasshouses and other protected environments (Tullett *et al.*, 2004; Hatherley *et al.*, 2005; Hughes *et al.*, 2009; Bale, 2011).

As well as not being an establishment risk, a glasshouse invertebrate biological control agent must be effective in order to warrant application to control a pest species (McClay and Balciunas, 2005). Behavioural responses to temperature are an important component in the selection criteria of an invertebrate biocontrol agent, as the thermal thresholds of a poikilothermic organism dictate the minimum and maximum temperatures at which activity and life processes can proceed (Huey and Kingsolver, 1989). These parameters provide a preliminary indication of whether a species is likely to survive winter conditions outside a glasshouse environment, and demonstrate the extent to which the species is able to effectively exert control over a horticultural pest in an open release scenario.

The critical thermal minima (CT_{min}) and chill coma are non-lethal measures of the lowest temperatures at which an invertebrate can perform motile tasks. The CT_{min} is the lowest

temperature at which it is possible for a species to walk in a coordinated manner (Cowles and Bogert, 1944). The organism can retain use of its appendages at temperatures below the CT_{min}, but on entering chill coma, all movement ceases (Mellanby, 1939). Providing the exposure temperature increases, invertebrates are usually able to regain use of limbs (chill coma recovery) and eventually walk in a coordinated manner (activity recovery). However, if the temperature remains below the chill coma temperature for an extended period, the invertebrate may be killed by the low temperature exposure (Mellanby, 1939).

In contrast, upper thermal thresholds are measures of high temperatures resulting in an invertebrate losing the ability to walk in a coordinated manner (CT_{max}) and use of appendages (heat coma) (Mellanby, 1939; Cowles and Bogert, 1944). Previous studies of a variety of invertebrates have shown that heat coma is also effectively the upper lethal limit (Hazell *et al.*, 2010; Hughes *et al.*, 2010a,b). Whilst recovery from CT_{max} has been recorded in *Atta sexdens rubropilosa* Forel (Hymenoptera: Formicidae), there was no significant difference between the temperatures at which the predatory mites *Phytoseiulus macropilis* Banks and *Phytoseiulus persimilis* Athias-Henriot (Acari: Phytoseiidae), and the herbivorous mite *Tetranychus urticae* Koch (Acari: Tetranychidae) encountered CT_{max} and heat coma (Ribiero *et al.*, 2012; Coombs and Bale, 2013).

This study examines the thermal behavioural thresholds of *Balaustium hernandezi* von Heyden (Acari: Erythraeidae), a candidate glasshouse biological control agent of the phytophagous crop pest *T. urticae*. *Balaustium hernandezi* is a recently described species, originating from Spain. Although little is known about this particular species' thermal thresholds, the genus is known for its high temperature tolerance (Yoder *et al*., 2007a; 2008;

Hedges *et al.*, 2012). In order to be classed as an effective predator, the activity thresholds and walking speeds of the predator should broadly reflect those of its prey.

7.3. Materials and Methods

7.3.1. Cultures

Rearing methods of the mites are described in Chapter 2.

7.3.2. Experimental system

The experimental system developed by Hazell *et al.* (2008) has previously been used to assess the thermal activity thresholds of a variety of invertebrate species (Hazell *et al.*, 2010; Hughes *et al.*, 2010a,b; Coombs and Bale, 2013). Mites were placed in an arena with a diameter of 25mm and a depth of 7.5mm in an aluminium block that allowed the passage of cooled or heated fluids from a connected alcohol bath (Haake Phoenix 11 P2, Thermo Electron Corp., Germany). The arena was covered with a clear plastic Petri dish, and the walls of the arena coated with Fluon (Blades Biological, UK) to prevent the escape of the mites. A type K thermocouple connected to a thermometer (Tecpel Advanced Digital Thermometer DTM-315, Heatmiser, UK) was inserted into the arena wall, which recorded the temperature throughout the experiment.

CT_{min}, chill coma and recovery experiments were conducted in a controlled environment room at 10˚C. CT_{max} and heat coma experiments were conducted in a similar room at 23˚C. The thermal activity thresholds of the mites were recorded for retrospective analysis using an Infinity 1 digital camera (Lumenera, Ottowa, Canada), with a 10x macro lens (MLH-10X,

Computar, CBC Corp., New York, USA). Videos were recorded using Studio Capture, and analysed using Studio Player and Studio Measure (Studio86Designs, Lutterworth, UK).

In order to compare predator and prey, lower and upper critical thermal limit data for *T. urticae* from Coombs and Bale (2013) were used.

7.3.3. Calibration

The arena and actual insect temperatures were calibrated as in section 4.3.3. All experimental results were then calibrated using the results of the linear regression.

7.3.4. CT_{min} and chill coma

A sample of six mites were transferred into the arena, and the temperature was reduced at a rate of 0.2°C min^{-1} from 25 to -4°C, as preliminary experiments demonstrated 100% of *B. hernandezi* entered chill coma prior to this temperature. CT_{min} was recorded as the temperature at which each individual made a final coordinated ambulatory movement, and chill coma as temperature at which the final twitch of an appendage was made. Thirty individuals were monitored for entry into CT_{min} and chill coma.

7.3.5. Chill coma and activity recovery

Thirty fresh individuals were used to measure chill coma and activity recovery. Samples of six mites were transferred into the arena, and the temperature was reduced from 25 to -4°C at a rate of 0.5°C min^{-1}. The arena was held at -4°C for 10 min to ensure all individuals had entered chill coma, and then returned back to 25°C at a rate of 0.2°C min^{-1}. As the

temperature increased, chill coma recovery was recorded as the temperature of the earliest twitch of an appendage, and activity recovery as the temperature at which the mite resumed coordinated locomotion.

7.3.6. CT_{max} and heat coma

Thirty individuals of each species were observed for entry into CT_{max} and heat coma. Samples of six mites were transferred into the arena, and the temperature was increased from 25 to 60˚C at a rate of 0.2°C min^{-1}. CT_{max} was measured as the temperature at which each individual made a final coordinated ambulation, and heat coma as the temperature at which the final twitch of an appendage was made. As entry into heat coma was effectively the upper lethal temperature, it was not possible to record recovery from heat coma.

7.3.7. Walking speed

Thirty individuals were observed to determine the mean walking speed at a range of temperatures. Samples of three mites were transferred into the arena. The temperature of the arena was raised from 25 to 30°C, and individuals were recorded for 10 min. The temperature of the arena was then lowered at 5°C intervals to 0°C at 0.5°C min^{-1}, holding the mites at each progressively lower exposure temperatures for 10 min. The first 5 min of each exposure were allocated in order to negate any lag in temperature between the arena and the mites, therefore only the final 5 min of each exposure was analysed. The distance each mite travelled over the 5 min was measured, from which mean walking speed could be calculated.

7.3.8. Statistical Analysis

All statistical analyses were made using the statistical package Minitab 15 (Minitab Ltd., Coventry, UK). *Tetranychus urticae* data were sourced from Coombs and Bale (2013).

The critical thermal minima and maxima, entry into chill and heat coma and resumption of activity were initially analysed using distribution ID plots, which confirmed the most appropriate distribution to utilise in further analysis of the results. In all cases the Weibull distribution was considered the best fitting and therefore most suitable, and is a commonly utilised distribution in the analyses of life data (Clifford Cohen, 1965). Upon ascertaining that the data were normally distributed, parametric distribution analyses could be performed using the Weibull distribution. These analyses identify differences in both shape and scale of the data between each species. Significant differences within the data were confirmed using one-way ANOVAs and Tukey's HSD *post-hoc* tests.

The walking speed data were not normally distributed; accordingly, it was determined the non-parametric Scheirer-Ray-Hare extension of the Kruskal-Wallis test was the most appropriate to analyse the data.

Results were considered significant when $p < 0.05$.

7.4. Results

7.4.1. Calibration

The difference between arena temperature and actual mite temperature varied between species and arena, and the resulting regression equations were used to analyse corresponding data (Fig. 7.1).

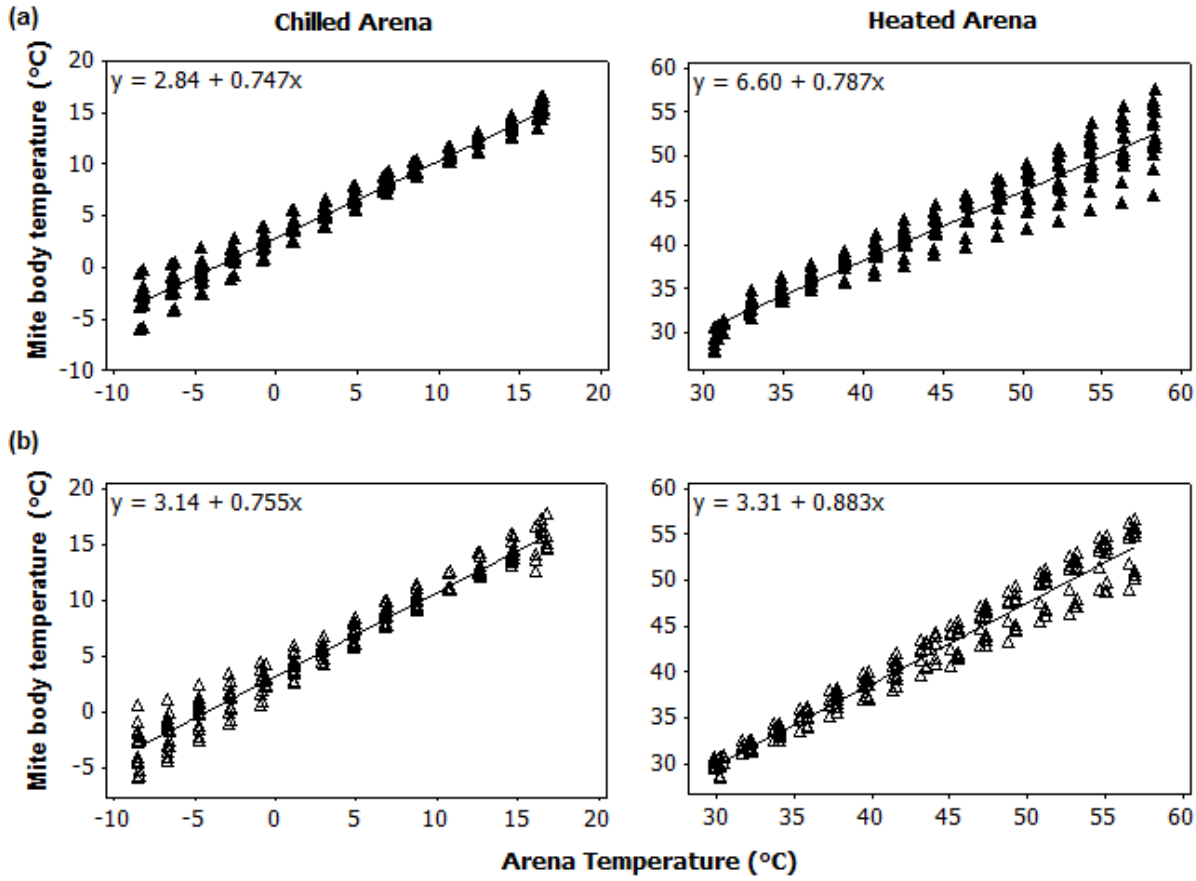

Figure 7.1. Regressions of arena temperature against body temperature for the calibration of lower and upper thermal thresholds in the chilled and heated arenas respectively. Calibrations were undertaken for two cohorts of *Balaustium hernandezi*: **(a)** adults and **(b)** larvae.

7.4.2. CT_{min} and chill coma

There was a significant difference between the temperatures at which each species reached CT_{min} ($p < 0.001$; $\chi^2 = 58.58$; $df = 4$). *Balaustium hernandezi* adults and larvae were able to

retain ambulatory function to lower mean temperatures (5.9 and 8.1˚C respectively) than *T. urticae* (10.6˚C). The predatory mites encountered CT_{min} at a more narrow range of temperatures than the prey species (Table 7.1).

Table 7.1 Mean ± SE and range (in brackets) of temperatures (°C) at which each adult and larval *B. hernandezi* and adult *T. urticae* experienced CT_{min}, chill coma, chill coma recovery and activity recovery (source of *T. urticae* data: Coombs and Bale, 2013). Means followed by different letters are significantly different (Tukeys HSD, $p < 0.05$)

Species (cohort)	CT_{min}	Chill coma	Chill coma recovery	Activity recovery
Balaustium hernandezi **(adult ♀)**	5.9 ± 0.3 [a] (4.6 – 10.2)	-2.1 ± 0.1 [d] (-2.6 – -1.3)	0.4 ± 0.4 [e] (-2.7 – 12.0)	13.2 ± 0.3 [g] (11.1 – 15.6)
Balaustium hernandezi **(larvae)**	8.1 ± 0.6 [b] (5.3 – 14.2)	-1.7 ± 0.2 [d,e] (-3.4 – -0.2)	-0.5 ± 0.2 [e] (-3.5 – 1.2)	13.0 ± 0.2 [g] (10.9 – 17.3)
Tetranychus urticae **(adult ♀)**	10.6 ± 0.5 [c] (5.3 – 16.2)	10.3 ± 0.5 [c] (4.7 – 15.7)	12.2 ± 0.6 [f] (8.4 – 20.4)	12.8 ± 0.6 [f] (8.4 – 20.4)

Mean chill coma temperature was also significantly lower in *B. hernandezi* adults and larvae compared to *T. urticae* (-2.1, -1.7 and 10.3˚C respectively) ($p < 0.001$; $\chi^2 = 377.67$; $df = 4$). The range of temperatures at which both cohorts of *B. hernandezi* experienced chill coma was narrow (1.3 and 3.2˚C respectively) compared with a difference of 11.0˚C in *T. urticae* (Table 1). There was a significant difference between the mean temperatures at which *B. hernandezi* adults ($p < 0.001$; $F_{1,58} = 954.25$) and larvae ($p < 0.001$; $F_{1,58} = 214.0$) ceased ambulation and twitching of appendages; however, there was no significant difference between the temperatures at which *T. urticae* demonstrated these behaviours ($p = 0.610$; $F_{1,58} = 0.26$).

7.4.3. Chill coma and activity recovery

Adult and larval *B. hernandezi* recovered use of their limbs at a lower temperature than *T. urticae* (0.4, -0.4 and 12.2˚C respectively). There was a difference in the shape and scale of the distribution of chill coma recovery temperatures between the two species ($p < 0.001$; $\chi^2 = 453.74$; $df = 4$). The range of temperatures at which adult *B. hernandezi* recovered from chill coma spanned 14.7˚C, which was much greater than the range of temperatures where the species entered chill coma (Table 7.1). In comparison, larval *B. hernandezi* recovered across a range of 4.7˚C, similar to the range at which the cohort experienced chill coma (3.6˚C).

There was a significant difference between the temperatures at which *T. urticae* entered CT_{min} and chill coma, and the temperatures at which the species recovered use of its limbs and ambulation ($p = 0.006$; $F_{3,116} = 4.39$). *Post-hoc* tests revealed, however, that only the mean temperature at which the species entered chill coma was significantly different to the temperature at which ambulation was recovered; all other low temperature threshold behaviours occurred at similar temperatures.

In contrast, *B. hernandezi* demonstrated all four lower thermal threshold behaviours at significantly different temperatures ($p < 0.001$; $F_{3,104} = 402.29$). Activity recovery occurred at a higher temperature than chill coma recovery ($p < 0.001$; $F_{1,46} = 410.15$), and was not significantly different to the temperature at which *T. urticae* recovered ambulation (13.2 and 12.8˚C respectively) ($p = 0.651$; $F_{1,46} = 0.21$).

7.4.4. CT_{max} and heat coma

There was no significant difference between the temperatures at which adult or larval *B. hernandezi* and *T. urticae* entered CT_{max} (46.7, 46.2 and 47.2˚C respectively) ($p = 0.422$; $F_{2,87} = 0.87$). Although both species ceased movement at similar temperatures, adult *B. hernandezi* entered CT_{max} in the narrowest range of temperatures compared to larvae and *T. urticae* (Table 7.2). Larval *B. hernandezi* survived the effects of increasing temperature beyond the limits of the adults (50.9 compared to 47.9˚C respectively).

Table 7.2 Mean ± SE and range (in brackets) of temperatures (°C) at which each adult and larval *B. hernandezi* and adult *T. urticae* experienced CT_{max} and heat coma (source of *T. urticae* data: Coombs and Bale, 2013). Means followed by different letters are significantly different (Tukeys HSD, $p < 0.05$)

Species (cohort)	CT_{max}	Heat coma
Balaustium hernandezi **(adult ♀)**	46.7 ± 0.1 ^{h} (45.3 – 47.9)	46.8 ± 0.1 ^{h} (46.0 – 47.9)
Balaustium hernandezi **(larvae)**	46.3 ± 0.4 ^{h} (43.0 – 50.9)	46.7 ± 0.3 ^{h} (44.4 – 50.9)
Tetranychus urticae **(adult ♀)**	47.3 ± 0.9 ^{h} (39.4 – 54.9)	48.7 ± 0.7 ^{i} (43.8 – 55.2)

While there was no significant difference between the temperatures at which *T. urticae* ceased ambulation and twitching of its appendages ($p = 0.251$; $F_{1,58} = 1.35$), the herbivore retained control of its limbs to higher temperatures than *B. hernandezi* ($p < 0.001$; $\chi^2 = 372.51$; $df = 4$). There was no significant difference between CT_{max} and heat coma thresholds in adult ($p = 0.231$; $F_{1,58} = 1.46$) or larval *B. hernandezi* ($p = 0.395$; $F_{1,58} = 0.73$). The range of temperatures at which adult and larval *B. hernandezi* experienced heat coma was narrower than *T. urticae* (Table 7.2).

7.4.5. Walking speed

There was a significant difference between the walking speeds of *B. hernandezi* and *T. urticae* ($p < 0.001$; $H = 18.80$; $df = 2$). Adult *B. hernandezi* were able to move significantly faster than larvae and *T. urticae* at all temperature intervals between 10 and 30˚C, except for 25˚C (Fig. 7.1). Although adult *B. hernandezi* appear to move at a higher speed at 5˚C compared with larvae and *T. urticae*, examination of the Bonferroni 95% confidence intervals showed no significant difference. There was no significant difference between the walking speed of larval *B. hernandezi* and *T. urticae* at any temperature ($p = 0.697$; $H = 0.151$; $df = 1$).

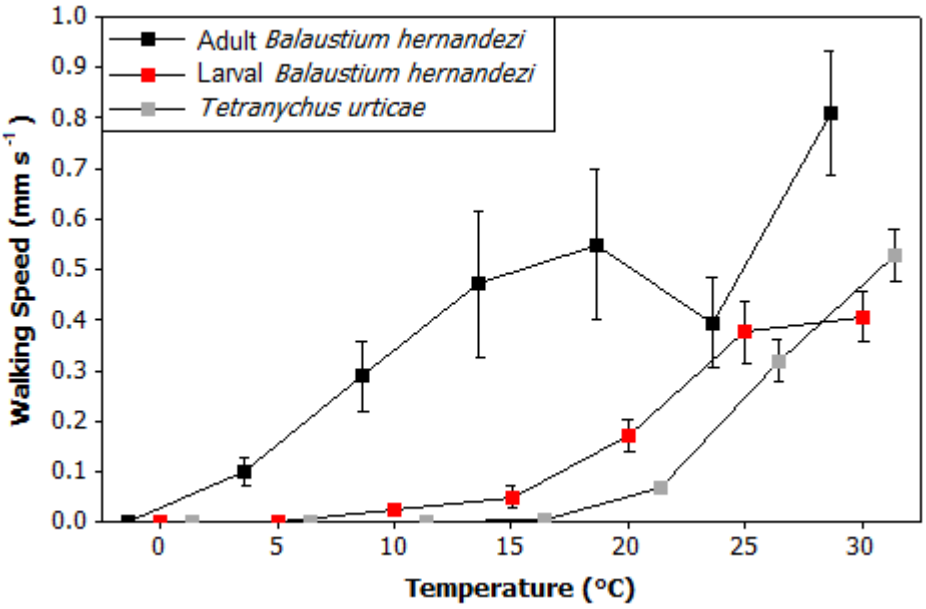

Figure 7.2. Mean (± SE) walking speed (mm s^{-1}) of adult and larval *B. hernandezi*, and *T. urticae* at different temperature intervals (source of *T. urticae* data: Coombs and Bale, 2013)

7.5. Discussion

Invertebrate behaviour is dictated by temperature, and as such, the study of thermal thresholds has particular importance within the assessment of a species' suitability for biological control.

Thermal activity thresholds demonstrate the limits of a poikilothermic organism's ambulation ability, which will have direct influence on life processes such as predator avoidance, oviposition, foraging and prey consumption (Crist and MacMahon, 1991; Gotoh *et al.*, 2004; Ahnesjö and Forsman, 2006; Berger *et al.*, 2008). An augmentative biological control agent intended for use in a temperate region, such as *B. hernandezi*, should preferably retain locomotor function at temperatures which render target prey immobile, and match or exceed the speed of prey movement.

The data indicates *B. hernandezi* would make an effective augmentative biological control agent in a northern European climate as the species maintained coordinated movement to temperatures below that of its prey, and would therefore be able to forage when environmental conditions have rendered *T. urticae* immobile. There are, however, wider safety implications as the mite can retain use of its limbs at sub-zero temperatures, and recovers from chill coma at 0.4˚C. As the CT_{min} and chill coma temperatures are usually higher than the lower lethal limit of the species, the data indicates that *B. hernandezi* may be able to survive colder conditions (Mellanby, 1939). If *B. hernandezi* has a robust cold tolerance strategy, and can survive a typical northern European winter and reproduce successfully, long-term establishment is possible. Maintaining ambulation at low temperatures also maximises the opportunity mites have to seek refugia, such as tree bark, pedicels of fruit or leaf litter, at the onset of more adverse conditions (Veerman, 1992). The refugia site offers a more stable microclimate, protecting the individual from the adverse conditions of winter, and thus increases the chance of survival to the following spring (Danks, 2002). The combination of these factors may render the species unsuitable for use as an

augmentative biological control agent due to the chance of glasshouse escapee survival and establishment (Hatherly *et al.*, 2005; Coombs and Bale, unpublished data).

The range of 7 to 12°C has previously been noted to slow the movement of *Balaustium* sp. nr. *putmani*, which aggregate at lower temperatures (Yoder *et al*., 2010). This behaviour has been hypothesized as a mechanism to alleviate water stress, and has been observed in other acarine species such as *Dermatophagoides farinae* Hughes (Acari: Pyroglyphidae) and *Alaskozetes antarcticus* Michael (Acari: Cryptostigmata) (Block and Convey, 1995; Glass *et al.*, 1998; Benoit *et al.*, 2008). The cluster reduces the exposed surface area for each individual, and the internal area of the aggregation will have a higher relative humidity, reducing the risk of desiccation (Lockwood and Story, 1986). As the mites were studied in very low densities in the thermal threshold and walking speed experiments, no aggregation behaviour was recorded. Although it is likely that the number of escapees from a glasshouse environment would be low, clustering behaviour may aid survival in the external environment and subsequently aid establishment of the species.

Balaustium hernandezi adults are able to secrete a defensive fluid from the urnulae, a pitted tubule structure posterior to the eyes (Mąkol *et al*., 2012). The fluid is spread over the mite body surface, and has been found to have several defensive functions in species of the genus *Balaustium*: as an alarm pheromone and as an allomone (Yoder *et al*., 2010). In addition, the urnulae-derived fluids have also been found to enhance the waterproofing of *Balaustium* sp. near *putmani*, which can survive brief exposures to 52˚C (Yoder *et al.*, 2007a; 2008). Water loss during increasing temperature and dehydration stress primarily arises from transpiration through the cuticle, and to a lesser extent through respiration (Gibbs *et al.*, 1997). The

waterproofing action of the fluids will slow desiccation, and protect the mite from the effects of extremes of temperature.

Balaustium hernandezi was able to resist the deleterious effects of increasing temperature to 47˚C, demonstrating a considerable tolerance of the effects of heating. The CT_{max} of many tropical arthropods is lower, for example: 34.9˚C in *Nilaparvata lugens* Stål (Homoptera: Delphacidae) (Piyaphongkul *et al.*, 2012); 40.0˚C in *Linepithema humile* Mayr (Hymenoptera: Formicidae) (Jumbam *et al.*, 2008); and 42.6˚C in *P. macropilis* (Coombs and Bale, 2013). A higher CT_{max} has previously been found to correlate with hotter native environments, albeit very weakly (Addo-Bediako *et al.*, 2000; Deutsch *et al.*, 2008). *Balaustium hernandezi* is found at lower latitudes, and has a Mediterranean native distribution (Mąkol *et al*., 2012). The xeric nature of the origins of *B. hernandezi* may account for the increased tolerance of high temperatures. A comparison between several *Drosophila mimica* Hardy (Diptera: Drosophilidae) populations demonstrated that those individuals with dry distributions were more resistant to desiccation than their mesic conspecifics (Eckstrand and Richardson, 1980).

Species with a lower surface area to volume ratio can demonstrate a more protracted resistance of the deleterious effects of increasing temperatures (Le Lann *et al*., 2011). There is a substantial difference in size between the species, whereby adult *B. hernandezi* exceed 2mm in length, and adult *T. urticae* have an idiosoma length of ~420μm (Mąkol *et al*., 2012; Coombs and Bale, 2013). Despite the disparity, there was no difference between the temperatures at which *B. hernandezi* and *T. urticae* were no longer able to walk in a coordinated manner due to increasing temperature. The data indicate that *B. hernandezi* would

be an effective biological control agent in regions where the temperature reaches 40˚C as it can retain locomotor function to the same extent as *T. urticae*.

Several species within the genus *Balaustium* have been observed as fast moving (Yoder *et al.*, 2006; 2007b; 2010; Cadogan and Laing, 1977). The walking speed trial demonstrated that adult *B. hernandezi* are able to move significantly more quickly than larvae and *T. urticae*. Speed of movement increased up to 30°C, although adult walking speed fell to mirror that of larvae and *T. urticae* at 25°C. Investigation of *Balaustium* sp. nr. *putmani* has demonstrated that the species is motivated by the presence of honeydew volatile cues indicating the presence of prey species (Yoder *et al.*, 2010). The walking speeds of *B. hernandezi* may be increased by the presence of herbivore-induced plant volatiles, released in response to feeding damage, which are known to elicit foraging responses in other predatory mites such as *P. persimilis*, *Neoseiulus womersleyi* Schicha and *Neoseiulus californicus* McGregor (Acari: Phytoseiidae) (Dicke *et al.*, 1999; Shimoda *et al.*, 2005; Ishiwari *et al*., 2007). As both cohorts of *B. hernandezi* matched or exceeded the walking speed of *T. urticae* without the presence of volatile cues in the arena, the species should make an effective biological control agent of *T. urticae*.

There is no Europe-wide legislation to regulate the release of invertebrate biocontrol agents, resulting in a difference in the licensing process in different European countries, although a number of countries now operate under a more standardised process, including the UK and the Netherlands (Bale, 2011). *Balaustium hernandezi* is a recently described species, and to date populations of the species have only been found in the Almeria region of Spain (Mąkol *et al*., 2012). Invertebrate biological control agents are being applied to at least 20,000 hectares

of glasshouses in Spain (Pilkington *et al.*, 2010). As a native species, *B. hernandezi* could be applied to either glasshouse or open field environments as an effective spider mite predator, without contravening any biological control regulations.

The majority of previous non-native biocontrol agents released into glasshouses across northern Europe have originated from outside of the EU; for example, *P. persimilis* and *Amblyseius swirskii* Athias-Henriot (Acari: Phytoseiidae) are sourced from Chile and Israel respectively (Cock *et al.*, 2010). Signatories of the 'Convention on Biological Diversity' are committed to Access and Benefit Sharing (ABS), where countries hold sovereign rights to their own genetic resources. Twenty of the largest commercial producers of biological control agents are located within the EU, and will be affected by the implementation of ABS: ABS increases the cost of investigating exotic species, and may prevent it entirely (van Lenteren *et al.*, 2011). Biological control companies located within the EU region may in future focus more attention on native EU species such as *B. hernandezi*. However, if the full European distribution of the species is unknown, it is impossible to know in which countries *B. hernandezi* is non-native and would therefore require regulation. Data collected on the thermal biology of the species indicate that *B. hernandezi* is likely to survive a typical northern European winter, and therefore is unsuitable for release in countries outside of its native distribution (Coombs and Bale, unpublished data).

The data demonstrate that *B. hernandezi* has the potential to be an effective biological control agent in Spain, as its thermal behavioural thresholds mirror or exceed those of the target prey, *T. urticae*. However, as *B. hernandezi* can maintain motility below 10°C and some movement at sub-zero temperatures, further investigation is required to identify potential for

establishment in northern European countries without native populations. Investigation of the thermal thresholds has indicated a significant potential for dispersal of escapees outside of a glasshouse during winter conditions.

CHAPTER 8

General Discussion

Tetranychus urticae Koch (Acari: Tetranychidae) is a highly polyphagous herbivore, and is known to feed on 1,100 plant species including economically important crops such as tomato and maize (Grbić *et al.*, 2011). The species is able to rapidly develop resistance against pesticides through point mutations, metabolic processes and behavioural responses (Georghiou, 1972). In confronting the challenge of pest resistance against chemicals, and the growing constrictions on chemical use, the application of natural enemies is a commercially viable alternative (Isman, 2006; Birch *et al*., 2011; Hillocks, 2012).

Biological control is considered an environmentally friendly alternative to the application of pesticides, and more than 7000 introductions of 2700 non-native biological control agents have been made worldwide with few reported problems (Cock *et al*., 2010). The glasshouse biological control agent *Phytoseiulus persimilis* Athis-Henriot (Acari: Phytoseiidae) has been used in over twenty countries since 1968 to combat *T. urticae* (Cock *et al.*, 2010). The widespread success of agents such as *P. persimilis* provokes the question: why should users of glasshouse biological control invest in new biological control agents?

A candidate biological control agent must have the capacity to efficiently exert control over a pest population. *Phytoseiulus persimilis* has been the principal glasshouse biological control agent of *T. urticae* for several decades; however, tradition will not preclude the use of a new commercially reared agent should there be sufficient evidence to show it is more effective in reducing pest populations. *Encarsia formosa* Gahan (Hymenoptera: Aphelinidae) has been

used in glasshouses in 20 countries since 1926, and was the primary biological control agent of glasshouse whitefly *Trialeurodes vaporariorum* Westwood (Homoptera: Aleyrodidae) (Cock *et al.*, 2010). In 2005 *Amblyseius swirskii* Athias-Henriot (Acari: Phytoseiidae) was released on the market as a glasshouse biological control agent of *T. vaporariorum*, *Bemesia tabaci* Gennadius (Homoptera: Aleyrodidae), *Frankliniella occidentalis* Pergande (Thysanoptera: Thripidae) and *T. urticae*, and is already used in 20 countries (Cock *et al.*, 2010). The predator also appears to be unaffected by glandular trichomes, which have been known to hamper the efficacy of other acarid biological control agents (Walter, 1996; Cedola *et al.*, 2001; Buitenhuis *et al.*, 2014). The increased efficacy of the agent has resulted in quick adoption into commercial horticultural systems.

Before a candidate glasshouse biological control agent can be released, many countries, including the UK, require an environmental risk assessment to be performed. If a candidate agent is found to possess the capacity for dispersal and establishment upon escape of a glasshouse environment, the species is unlikely to be licensed for release. Increased understanding of the cold tolerance strategies of a biological control agent have slowly changed the experimental methodologies examining the risk of establishment in a non-native environment (van Lenteren *et al*., 2003, 2006; Hatherly *et al.*, 2005a; Hazell *et al.*, 2008). Climate matching was once utilised in the selection of a glasshouse biological control agent, whereby the native distribution was relied on as an indicator of whether a candidate agent would survive winter in a temperate climate (van Lenteren *et al*., 2006). *Neoseiulus californicus* McGregor (Acari: Phytoseiidae) has an arid, semi tropical and Mediterranean distribution, and has been shown to rapidly reduce *T. urticae* infestations (Raworth *et al.*, 1994; McMurtry and Croft, 1997; Greco *et al*., 2005). The principles of climate matching

would suggest it is an ideal agent for release in glasshouses in the UK, however, wild *N. californicus* populations were identified in orchards following introductions into UK glasshouses (Jolly, 2000). The virulent spread of invasive species such as *Harmonia axyridis* Pallas (Coleoptera: Coccinellidae), which has spread to seven countries where no deliberate release was made has highlighted the necessity of thorough investigation prior to release (Brown *et al.*, 2008).

The cold tolerance of two candidate biological control agents intended for use in glasshouses in northern Europe, *Phytoseiulus macropilis* Banks (Acari: Phytoseiidae) and *Balaustium hernandezi* Von Heyden (Acari: Erythraeidae), was assessed through a combination of laboratory and field experiments. The experiments were designed to investigate the risk of establishment in northern European winters. Where cold hardiness was limited, an investigation into the efficacy of the species was undertaken.

8.1. *Phytoseiulus macropilis*

The combination of thermal biology and threshold data indicates that *P. macropilis* presents a low risk of establishment in the UK and regions with a similar climate. *Phytoseiulus macropilis* demonstrated high mortality at relatively high temperatures during laboratory experiments, and quickly increasing mortality through field trial exposures. The mites were unable to survive through an acclimation regime of 7d at 10°C previously used to assess acclimation potential in candidate biological control agents, suggesting an accumulation of lethal chilling injuries at relatively mild temperatures (Allen, 2010; Hughes *et al.*, 2009). No diapause trait has yet been identified in the genus *Phytoseiulus*, and *P. macropilis* did not demonstrate any indication of entering the state in two generations of cool, short day

conditions (15°C; 11:13LD) (Veerman, 1992). In all experiments, adults demonstrated a higher degree of cold hardiness than the larvae. The combination of all the thermal biology results implies that *P. macropilis* can be classed as a chill susceptible species, and is therefore unlikely to survive a typical UK winter (Bale, 1993).

The lower thermal threshold data supported the use of *P. macropilis* as a glasshouse biological control agent in temperate climates. The loss of ambulatory ability at 8.2°C indicates that any escapees would have little dispersal ability away from a glasshouse environment during mild UK winter conditions. Movement was possible below temperatures which rendered *T. urticae* and the predatory mite *P. persimilis* immobile, suggesting that *P. macropilis* is able to forage at temperatures where both the prey and a competitor predator are unable to move. This finding was supported by results of the efficacy investigations. The predatory ability of *P. macropilis* was comparable with *P. persimilis* on French bean and tomato leaf surfaces, but exceeded it at 15°C.

Biological control agents need to be both safe and effective in order to merit commercial release (McClay and Balciunas, 2005). Survival on tomato leaf surfaces was lower for both predatory phytoseiids compared to survival on French bean; however, *P. macropilis* was no more hindered in its foraging than *P. persimilis*. Overall performance of *P. macropilis* was not significantly different to *P. persimilis*, which has successfully been used a glasshouse biological control agent since the 1960s, on any plant surface nor any temperature between 17.5 and 30°C. The data implies *P. macropilis* will make an effective glasshouse biological control agent of *T. urticae* in northern European glasshouses, and may offer an advantage over *P. persimilis* at lower glasshouse temperatures.

8.2. *Balaustium hernandezi*

Investigation into the cold tolerance of *B. hernandezi* demonstrated a high capacity to tolerate UK winter temperatures. The predatory mite was able to withstand prolonged exposures to relatively high temperatures in the laboratory and field. Adults demonstrated a higher degree of cold hardiness in laboratory and field trials, with the exception of lethal time at -5°C. Interestingly, the results suggest differing cold hardiness strategies between the two cohorts: there was no difference between the $LTemp_{50}$ and supercooling point of the adults, implying they are strongly chill tolerant (Bale, 1993). In contrast, there is a large difference between the $LTemp_{50}$ and supercooling point of the larvae, confirming the cohort as chill susceptible (Bale, 1993). The extended adult $LTime_{50}$ at 5°C demonstrated a robust endurance of chronic exposure to temperatures likely to be experienced in a UK winter. The $LTime_{90}$ of *B. hernandezi* larvae was prolonged at -5°C compared to the adults, which may be due to the significantly depressed supercooling point.

The ecological relevance of these measures was tested through field trials, which confirmed the cold tolerance of *B. hernandezi*. During the 2011 field trial, *B. hernandezi* samples were exposed to UK winter temperatures for over four months without 100% mortality occurring in the field. The 2012 field trial elicited 100% mortality in the field after eight weeks. Several species of *Balaustium* are known to overwinter at the egg stage, and if *B. hernandezi* possesses a diapause trait, it will only aid the survival and establishment of the species in the UK and similar climates (Putman, 1970; Belozerov, 2008a).

Lower thermal threshold data indicated an ability to maintain movement at low temperatures, which would assist the mite in dispersing away from a glasshouse environment during typical

UK winter conditions. The data indicates that *B. hernandezi* is unsuitable for commercial release in northern European temperature climates, such as the UK.

During heating experiments, *B. hernandezi* demonstrated a substantial capacity to tolerate the deleterious effects of high temperatures. This characteristic has been noted in previous studies of other species within the genus *Balaustium*, and mites have been observed to survive brief exposures to 52°C (Yoder, 2007a). The substantial heat tolerance of the species supports the augmentation of *B. hernandezi* within its native distribution.

8.3. Conclusions

There is a strong correlation between $LTime_{50}$ at 5°C and maximum field trial survival times, as has been highlighted in previous examinations of risk of establishment posed by candidate invertebrate biological control agents (Hatherly *et al*., 2005a). The *P. macropilis* and *B. hernandezi* field trial and lethal time at 5°C data supports the use of this correlation as a tool in the selection of glasshouse biological control agents suitable for use in northern European climates. The laboratory measure of lethal time at 5°C could be used as a cost effective, safe and quick method to streamline the glasshouse biological control agent selection and licensing processes.

In using the more conservative maximum field trial survival of each candidate glasshouse biological control agent, *P. macropilis* falls within the low risk category, whereas *B. hernandezi* presents a high risk of establishment in a UK winter (Fig 8.1). *Phytoseiulus macropilis* falls within the same category of risk as *A. swirskii*, which was licensed for use as a glasshouse biological control agent in the UK in 2006 (Allen, 2010). As the cold tolerance

profile of *P. macropilis* is similar to *A. swirskii*, and the predatory performance comparable with *P. persimilis*, the species meets the safety and efficacy requirements of a successful biological control agent (McClay and Balciunas, 2005). In contrast, the field trial survival and $LTime_{50}$ of *B. hernandezi* are similar to those of *Dicyphus hesperus* Knight (Hemiptera: Miridae) and *N. californicus*. The discovery of naturalised populations of *N. californicus* in orchards subsequent to application in UK glasshouses implies that species with similar cold tolerance profiles, such as *B. hernandezi*, are unsuitable for release in temperate northern Europe (Jolly, 2000).

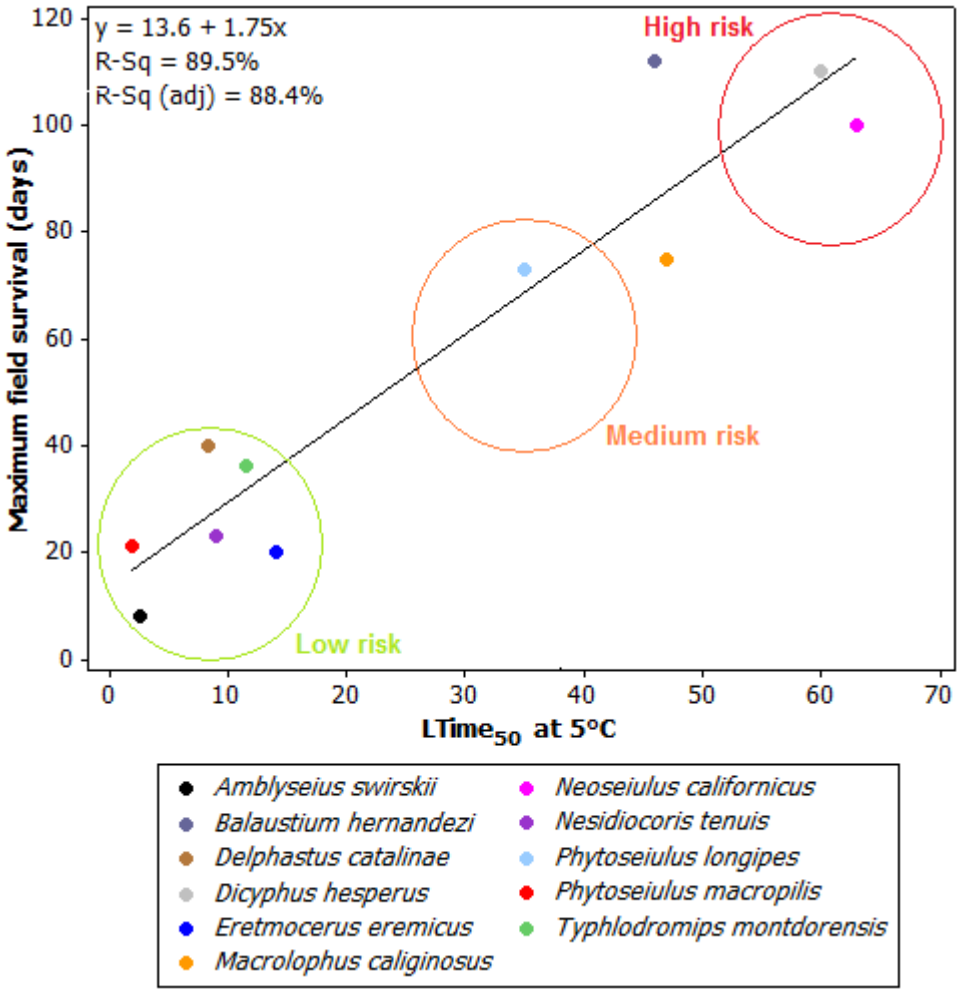

Figure 8.1. Correlation between $LTime_{50}$ at 5°C and maximum unfed field survival time of several biological control agents in low, medium and high risk categories. Sources of data: *Amblyseius swirskii* (Allen, 2010); *Delphastus catalinae* (Tullett, 2002); *Dicyphus hesperus* (Hatherley *et al.*, 2008); *Eretmocerus eremicus* (Tullett *et al.*, 2004); *Macrolophus calignosus* (Hart *et al.*, 2002a); *Neoseiulus californicus* (Hart *et al.*, 2002b); *Nesidiocoris tenuis* (Hughes *et al.*, 2009); *Phytoseiulus longipes* (Allen, 2010); *Typhlodromips montdorensis* (Hatherley *et al.*, 2004).

The results provide further support for the measurement of thermal activity thresholds, which have been used to assess the dispersal potential of several candidate biological control agents (Allen, 2010; Hughes *et al.*, 2010a,b). Previous studies have also used the thermal activity thresholds as an indication of the efficacy of agents in the glasshouse; however, it is hoped the plant surface and stem crossing data demonstrate the merit of conducting discrete efficacy experiments at different temperature intervals likely to be experienced in a glasshouse environment.

8.4. Legislation

The use of novel glasshouse biological control agents in northern European countries may be delayed or prevented entirely by Access and Benefit Sharing (ABS) legislation. Signatories of the Convention on Biological Diversity have been granted sovereign rights over genetic material within the country, and there is growing concern that access to potential biological control agents will be limited or stopped entirely (Hunt *et al.*, 2008; Cock *et al.*, 2010). The thermal biology, activity thresholds and efficacy results demonstrate that *P. macropilis* presents a low risk of establishment within northern European environments, and exerts control over pest populations at a greater range of temperatures compared with the current market leader, *P. persimilis*. Should the application of Access and Benefit Sharing legislation escalate, candidate agents with tropical distributions such as *P. macropilis* are unlikely to be identified, the cost of biological control will increase, and the use of this sustainable and environmentally friendly technology will decline (Cock *et al.*, 2010).

8.5. Future directions for research

There are further opportunities for research into the use of *P. macropilis* and *B. hernandezi* as biological control agents.

Phytoseiulus macropilis has a very low cold hardiness capacity, and is unlikely to present a risk of establishment to the UK. This presents an opportunity for further research into the release of the predator into open field crop systems during the summer months, as winter temperatures would act as a natural barrier to persistence. The inability to persist from winter to winter would decrease any impacts on non-target species.

Balaustium hernandezi presents a considerable capacity to survive typical UK winters, and therefore the risk of escapee establishment is too high to permit authorisation of a license to release the species in temperate northern European glasshouses. However, commercial production of the mite could be used to augment natural populations within the native distribution. Thus far, *B. hernandezi* has been found in the Almeria region of Spain, which is the primary producer of strawberries in Europe, and a principal worldwide producer of tomato (Mąkol *et al.*, 2012; García-Mari and González-Zamora, 1999; Stansly *et al.*, 2004). *Balaustium hernandezi* is capable of withstanding the high summer temperatures of the region, and would survive in glasshouses, polytunnels and open field situations alike. Further investigation into the native distribution of the species and its host range would be required in order to identify the extent of any negative non-target effects.

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APPENDIX 1

Coombs, M. R., Bale, J.S. 2012. Comparison of thermal thresholds of the spider mite predators *Phytoseiulus macropilis* and *Phytoseiulus persimilis* (Acari: Phytoseiidae). *Experimental and Applied Acarology*. DOI 10.1007/s10493-012-9619-9