Thermal biology and establishment potential of two non-native candidate biological control agents, *Nesidiocoris tenuis* Reuter (Hemiptera: Miridae) and *Lysiphlebus testaceipes* (Cresson) (Hymenoptera: Braconidae, Aphidiinae), in the U.K.

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ABSTRACT

Nesidiocoris tenuis Reuter (Hemiptera: Miridae) and *Lysiphlebus testaceipes* (Cresson) (Hymenoptera: Braconidae, Aphidiinae) are candidate biological control agents known to play an important role in the management of agricultural and horticultural pests in southern Europe. Through a series of laboratory and field assessments, this study investigates the establishment potential of these two species in cool temperate climates typical of northern Europe. Laboratory results demonstrated a low level of cold tolerance in *N. tenuis* with a developmental threshold of 12.9°C and no indication of ability to diapause. Field trials supported these findings with 100% mortality occurring after less than 4 weeks of winter field exposure. Collectively, these data suggest that *N. tenuis* is unlikely to establish outdoors in northern Europe and would therefore have little or no non-target effects on native species in such regions, thereby constituting a 'safe' candidate for release. Additionally, investigations into temperature-related thresholds indicated that *N. tenuis* would be an effective control agent against species with a similar activity profile to the two-spotted spider mite *Tetranychus urticae* Koch (Acari: Tetranychidae). *Lysiphlebus testaceipes* demonstrated a greater ability to tolerate cold than *N. tenuis* but there was no indication of ability to diapause. With a developmental threshold of 5.8°C, parasitoid larvae and pupae continued to develop during the 70 d of winter field trials yielding reproductively viable adults. With this level of cold tolerance and a host range in excess of 100 aphid species, including some known to overwinter in the UK and other temperate regions, it seems reasonable to predict that *L. testaceipes* would be able to establish in northern Europe. Thermal activity threshold investigations also indicated that *L. testaceipes* would constitute an effective control agent for pest species with similar activity profiles to *Aphis fabae* Scop. (Hemiptera: Aphididae) under a range of climatic conditions. These data are discussed in relation to current debate on the environmental risk assessment and regulatory system in Europe for the release of non-native biological control agents.

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CHAPTER 1

Introduction

1.1 BIOLOGICAL CONTROL

Chemicals have been used successfully for the control of pest species for thousands of years. The first known example of this was the use of sulphur by the Sumerians to control insects and mites in 2500 BC (Dent, 2000). Whilst the agricultural industry is still dominated by this form of pest control, concern about the impact of these substances on the environment and both consumer and producer health has resulted in increasing demand for alternative methods. These concerns, first highlighted in the book *Silent Spring* by Rachel Carson (Carson, 1963), have been further compounded by factors such as the accumulation of chemicals in the food chain (Bale *et al.*, 2008) and the emergence of pesticide resistance (Bale *et al.*, 2008; Pimentel, 2009). In addition to this, recent increases in the cost of pesticide development and registration have led to further emphasis being placed on the research and development of new methods of pest control (van Lenteren & Woets, 1988; van Lenteren & Manzaroli, 1999).

One alternative, biological control, has been utilised for over 100 years with varying degrees of success. This form of pest management encompasses the use of a range of natural substances and organisms for the control of pest species, usually through the manipulation of natural phenomena such as predation, parasitism or herbivory. Agents employed for biological control purposes include microorganisms such as viruses, fungi and bacteria, invertebrates such as insects, mites and nematodes, and behaviour modifying semiochemicals such as pheromones, although the latter group of compounds is not included in some definitions of biological control (e.g. DeBach, 1963).

1.2 TYPES OF BIOLOGICAL CONTROL

There are three main types of biological control: classical, augmentative and conservation control (van Lenteren, 2000a).

1.2.1 Classical control

Classical control involves the regulation of a pest species through the importation and release of its co-evolved natural enemy. As the target species tends to be an exotic, it often reaches higher densities in its novel surroundings due to favourable conditions such as the lack of predators or parasitoids (Caltagirone, 1981). In these situations, the release of the species' natural enemy can have a major impact on population numbers, often reducing pest densities to levels below those known to have an economic impact.

The first known example of this approach to pest control was recorded over 120 years ago (1888) when the Australian vedalia beetle, *Rodolia cardinalis* (Mulsant) (Coleoptera: Coccinellidae), was released into Californian citrus groves for the control of the exotic pest the cottony cushion scale, *Icerya purchasi* (Maskell) (Hemiptera: Margarodidae), a species that had earlier been transported to the US from Australia on *Acacia* plants (Ebeling, 1959). By the time *R. cardinalis* was released, *I. purchasi* was threatening to decimate the Californian citrus industry. However, such was the extent of the control achieved through the release of this predator, all infestations in the state had been destroyed by 1890 (DeBach & Rosen, 1991).

Although this form of biological control has been known to achieve desirable outcomes in terms of pest suppression, success is very much dependent on the environment into which the organisms are released. Control agents are more likely to achieve the permanent suppression of a pest population on perennial crops, such as forests and fruit plantations, where stable conditions favour the perpetuation of both the pest and the control agent (Bale *et al*., 2008). In annual crop systems, as a result of the regular harvesting of produce and habitat disturbance, such permanence is less likely.

There have been many documented benefits of this form of pest management, the main advantage being the high level of control that is achieved in the event of a successful release.

Furthermore, as control is often permanent due to the self-perpetuating nature of the agents, classical control can be an economically appealing option.

1.2.2 Augmentative control

Two distinct types of augmentative control have been recognised: inundative and seasonal inoculative release. These differ in the extent of control achieved through the release of natural enemies. Inundative control results in the immediate mortality of pest species through the periodic release of large numbers of laboratory-reared natural enemies (indigenous or native), the aim being to overwhelm pest populations resulting in an extent of pest suppression equivalent to that achieved through the use of pesticides (Bale *et al*., 2008). Here there is no expectation of long-term regulation as control is achieved only by the released organisms, not their offspring (van Lenteren, 2003). Seasonal inoculative control, however, is capable of achieving a level of pest suppression for a number of generations, generally for the duration of the growing season. This form of control has been used successfully for many years on agricultural crops and, although it was originally thought that biocontrol agents would not provide an adequate level of protection for the high value crops in glasshouses, it is now becoming an increasingly popular form of pest management within these enclosures (van Lenteren & Woets, 1988, Paulitz & Bélanger, 2001). Both inundative and inoculative control are particularly useful on annual crops, where the cropping method does not favour long-term establishment and pest-prey interactions.

The first example of inoculative control within a glasshouse environment involved the use of the parasitoid *Encarsia formosa* Gahan (Hymenoptera: Aphelinidae), in the 1920s, for the control of the glasshouse whitefly, *Trialeurodes vaporariorum* Westwood (Homoptera: Aleyrodidae). This proved to be so successful that, within a few years, the parasitoid was mass produced in Britain and shipped to many other parts of Europe as well as Canada, Australia and New Zealand (van Lenteren & Woets, 1988). In spite of this outstanding success, interest waned after the Second World War as a result of the development of pesticides (van Lenteren & Woets, 1988; Hoddle *et al.*, 1998). However, since the 1970s there has been resurgence in the use of this parasitoid (Hoddle *et al.*, 1998), with reports of its employment in nearly 5000 ha of glasshouses worldwide (van Lenteren, 1995).

1.2.3 Conservation control

Many pest populations can be managed effectively by enhancing the performance and abundance of natural enemies (Barbosa, 1998; Pickett & Bugg, 1998; Landis *et al*., 2000). This practice, termed conservation control, is possibly the oldest form of biological control based on cultural practices dating back many years (Kean *et al*., 2003). It involves the manipulation of the environment to enhance the survival, fecundity and longevity of native natural enemies for the control of indigenous and alien pest species (Landis *et al*., 2000).

As for classical control, conservation control is generally more successful in perennial as opposed to annual crop systems, as the seasonal harvest of annual crops may impede the ability of the arthropod predators (and to a lesser extent, parasitoids) to overwinter (Landis *et al*., 2000). This issue has led to the development of methods aimed at maintaining a predator presence throughout the winter, through the provisioning of suitable overwintering sites. Such methods of preservation and augmentation include the maintenance of hedges and construction of beetle banks to provide refuges and overwintering sites for polyphagous predators such as Carabid beetles (MacLeod, 2004), the provision of flower strips to attract predators such as syrphids and the availability of refugia outside the cropping area for times when the use of pesticides or heavy farm machinery is unavoidable. Whilst these methods are seldom used as a substitute for a more common form of pest control, the integration of such techniques into an existing pest control system can provide substantial benefits. For example, in instances where inundative or classical control is used, habitat management can maximise the persistence and effectiveness of the control agents (Landis *et al*., 2000). Alternatively, where chemical control is the preferred method, the integration of these techniques can reduce the number of pesticide applications.

1.3 BENEFITS OF BIOLOGICAL CONTROL

Approximately 40% of all organisms used in biocontrol are not native to the country or region into which they are released (Patrick de Clercq, pers. comm.). In this respect, they could be regarded as invasive species, at least potentially so. The damaging effects of invasive species on ecosystems are well documented (e.g. Pimentel *et al.*, 2005; Snyder & Evans, 2006; Strayer *et al*., 2006). However, with a proper pre-release evaluation system of

candidate control agents, biological control offers an important alternative to chemical control, with several unique benefits and without many of the adverse effects associated with pesticide or mechanical control.

With the advent of modern pesticides in the 1940s, the utilisation of arthropods as control agents was all but abandoned (Stinner, 1977; Cruz & Segarra, 1992), the general consensus being that chemical control was a more reliable and efficient means of controlling pest species. Whilst there was some truth in this, many of the negative opinions stemmed from early biocontrol programmes where the risks were less appreciated and there were a number of failures as a result of a trial and error approach to the selection of control agents. Today, a more predictive approach is taken, with candidate biocontrol agents undergoing rigorous preliminary studies to ensure their safety and efficacy prior to release.

In terms of the safety of this form of pest management, biocontrol has several advantages. Unlike chemical control, there are very few concerns regarding adverse effects to consumer and producer health, with no residues on produce or exposure of personnel to toxic chemicals. Also, in the absence of these toxins, there is no safety period during which re-entry into the treated area is prohibited. Thus, continuous harvesting is possible without compromising the health of the employees (van Lenteren, 2000b). Biocontrol also has considerable benefits from an environmental perspective. As the development and implementation of this form of pest management continue to increase, reliance on pesticides will decrease, leading to a reduction in the exposure of wildlife to chemical residues (Hoddle, 2003). In addition to this, biological control agents can be selected to have a high level of host specificity, a situation rarely achieved with pesticides (though more recent insecticides have greater specificity). This in itself can have substantial ecological benefits as it can help maintain natural enemy populations.

Due to the self-perpetuating nature of the agents in classical schemes and the ease with which these can be applied, biological control is often considered to be a more economically appealing form of pest management. Also, in the absence of phytotoxic effects on young plants, there is no premature flower and fruit abortion (van Lenteren, 2007), resulting in an increased yield. In addition to this, with the aforementioned increasing costs of pesticide development and registration, the economics of biocontrol development often compare favourably (Tisdell, 1990; Bale *et al*., 2008).

With regard to the efficacy of biocontrol, chemical control appears to be a more desirable option, with reports that only 10% of all biocontrol introductions achieve an adequate decrease in pest populations (Gurr *et al.*, 2000). However, it is important to remember that pesticides vary in their effectiveness over time and that the development of resistance can lead to the withdrawal of a pesticide from the market. In comparison, it is highly unlikely that resistance will develop to an invertebrate biocontrol agent. Thus, once an effective organism has been identified, its long-term use is assured. Another factor often cited as a limitation of biological pest control concerns the inability of a control agent to eradicate a pest completely. However, as highlighted by Bale *et al.* (2008), eradication is not always the optimal outcome. Whilst pesticides are capable of eliminating pest populations on a local scale, open environments are always susceptible to re-invasion, necessitating the need for further applications. Thus, the long term suppression of a pest species by an agent, below economic injury levels, is often a more desirable and economically viable option.

A comparative advantage of chemical control, in terms of efficacy, is that the reduction of pest populations occurs rapidly following application, whereas biological control agents often require time to achieve an adequate level of control, especially where establishment is required for success. Nevertheless, as control can be self-perpetuating in the event of a successful release, in the long term, this initial delay in pest suppression may be considered less important.

Finally, biological control is often the only option as it is the only viable method of reducing pest numbers over a large geographical range (Follett *et al*., 2000) and within inaccessible terrains (Howarth, 1991).

1.4 PEST CONTROL IN GLASSHOUSES

In classical and seasonal inoculative control, when introductions are made in outdoor environments such as fields, orchards and forests, it is essential that the control agent is able to establish and multiply for the required period for the control to be effective. However, in circumstances where biological control is used in glasshouses, the concerns are different. Due to the enclosed nature of this environment and the need to maintain a constant temperature and humidity for optimal crop growth, the survival of both the pest and the prey

species is generally guaranteed (van Lenteren & Woets, 1988; van Lenteren, 2000b). For this reason, whilst factors such as internal synchronisation, host specificity, reproductive potential and density responsiveness (van Lenteren & Manzaroli, 1999) are still crucial for successful glasshouse pest control, it is arguably of greater importance to determine what effect the species would have on the environment should it escape from the confines of the glass enclosure (Hart *et al*., 2002b).

In the past, the general assumption has been that insects originating from tropical, sub-tropical or Mediterranean regions would lack the necessary adaptations required for survival in cooler climates. Under this supposition 'climate origin' was used as a proxy for direct assessment of cold tolerance; thus it was thought sufficient for a risk assessment to include a comparison of the control agent's native climate and the climate into which it was to be introduced, the belief being that if the climates were sufficiently distinct, establishment potential would be low. However, such ideas have recently been called into question following the discovery of established populations of the glasshouse biological control agent *Neoseiulus californicus* McGregor (Acari: Phytoseiidae) outside glasshouse environments in the UK (Jolly, 2000), and frequent sightings in winter of the predatory mirid *Macrolophus caliginosus* Wagner (Hemiptera: Miridae) (Hart *et al*., 2002b). (It should be noted here that the identification of the species that has been marketed and released by the biocontrol industry as '*Macrolophus caliginosus*' is incorrect; however, this specific name is used here as it was the name under which the organism was supplied and released). Whilst there is currently no documentation of any negative impacts of the species on native fauna, regulations regarding the release of such agents have subsequently been tightened in several countries and it is now often necessary to provide a comprehensive report (an Environmental Risk Assessment) of the biology of the insect in question before release is permitted.

1.5 POTENTIAL IMPACTS OF BIOLOGICAL CONTROL INTRODUCTIONS

Over the past few years there has been increasing concern about the environmental effects of invasive exotics (Bale & Walters, 2001; Louda *et al*., 2003a, 2003b; van Lenteren *et al*., 2006). However, despite there being numerous well documented problems associated with the introduction of alien species to an environment, there have been relatively few reports of

such findings attributed to the release of biological control agents (Barratt *et al*., 1999). Nevertheless, introduced natural enemies have been documented to affect non-target species and the environment in a number of ways (Howarth, 1985, 1991; Simberloff & Stiling, 1996a,b) and it is probable that as the use of biological control continues to increase, the documentation of these effects will escalate (van Lenteren *et al.*, 2003).

To monitor the occurrence of any deleterious effects it is important that post-release studies, as well as assessing the efficacy of the control agent, should investigate the impact of the organism on non-target species and the environment. However, due to the vast number of species that have yet to be identified and described (see Raven & Johnson, 1992) and the myriad of effects that could occur, discerning the level of environmental damage caused by such introductions can be problematic (Pimentel *et al*., 2001). This is further complicated by the fact that records of ecosystem status prior to the introductions are seldom available for direct comparison.

To ensure the continuing success of this pest control method, it is essential that this situation is remedied and that a more predictive approach to biological control is sought.

1.5.1 Direct impacts on non-target species

Organisms introduced into an alien environment have the potential to affect the abundance of non-target species by directly attacking or competing with them. An example of this can be seen in the introduction of lepidopteran parasitoids to New Zealand. These parasitoids became so successful that they rapidly spread from their target habitats on farms and orchards, and now occur in native forests where they are parasitizing moth species endemic to the area (Russell, 1986). This case highlights the fact that agents released for biological control are not necessarily restricted to the habitat occupied by their target species (Follett *et al*., 2000), making it extremely difficult to predict their behaviour and potential impact once released.

However, although it has been estimated that the invasion of new areas by alien species has contributed to approximately 40% of the recorded extinctions worldwide (Caughley & Gunn, 1996), there are few known examples of deleterious impacts to non-target organisms as a result of the intentional release of biological control agents (van Lenteren *et al*., 2006).

Nevertheless, it is important to remain vigilant as this could be due to the aforementioned inadequacy in pre and post-release monitoring, as opposed to the true absence of non-target effects (Howarth, 1991).

1.5.2 Indirect impacts on non-target species

Knowledge of the possible impact of host-shifting on native species has led to greater emphasis being placed on the specificity of the control agents (McEvoy, 1996; Simberloff & Stiling, 1996a,b; Thomas & Willis 1998). However, investigations indicate that even host-specific organisms can have a deleterious effect on an ecosystem, as the successful control of the target species itself could, through a series of complex interactions, have non-target effects on native species (Zavaleta *et al.*, 2001; Pearson & Callaway, 2003). The probability of such an outcome is highly dependent on the trophic position of the target species within its niche. For example, if the target species is a herbivore, the control agent may be directly competing with its natural enemies, potentially leading to the decline or even exclusion of one of the competitors (Tilman, 1982).

A well known example of such indirect effects can be seen in the introduction of the *Myxoma* virus for the control of the European rabbit, *Oryctolagus cuniculus* (L.), in the UK. Although this introduction was almost certainly unsanctioned, it is still a widely cited example of the potentially deleterious consequence of biocontrol (Ehler, 2000). Whilst the introduction of the *Myxoma* virus had the desired effect, leading to a significant decline in rabbit numbers, it also resulted in the extinction of the large blue butterfly, *Maculinea rebeli Hir.* (Lepidoptera: Lycaenidae). This occurred as a consequence of the reliance of *M. rebeli* on the nests of the ant *Myrmica sabuleti* Meinert (Hymenoptera, Formicidae) for the development of their larvae. These ants were, in turn, reliant on the open habitats created by the grazing rabbits. Thus, through a series of interactions, the biocontrol of the rabbit indirectly resulted in the extinction of *M. rebeli* (see Moore, 1987). This case highlights the complex nature of community interactions and the importance of looking beyond host range when assessing possible risks associated with biocontrol releases.

1.6 REGULATION OF BIOLOGICAL CONTROL

There are four main categories of biological control agents: microorganisms, botanicals, semiochemicals and invertebrates. The key difference between these categories in terms of legislative restrictions is that, while the use of the first three (microorganisms, botanicals and semiochemicals) are governed by an EU Directive (EU Directive 91/414), at present, invertebrate use is not. In effect, this puts the suppliers of invertebrate control agents at a distinct advantage, as they do not have to fulfil the time consuming and costly guidelines set by a Directive, which would result in many companies being unable fund the registration of their products. Consequently, the use of invertebrates as a pest control option has become very successful commercially, with an annual turnover surpassing 150 million Euros (REBECA, www.rebeca-net.de). However, due to the growing concern about possible non-target effects of alien species on the environment, an increasing number of countries are implementing national legislation regulating the release of invertebrate control agents.

There are now several countries that require environmental risk assessment (ERA) data for non-native releases, but at present there is no consistency across borders or continents as to what data are necessary. In Australia, New Zealand, Canada and the USA, the guidelines are currently so strict that very few alien introductions are permitted (van Lenteren *et al*., 2006, Hunt *et al*., 2008) and in Norway, legislation prevents the import of any species that cannot be proven to be native to the country itself (Hatherly, 2005). In Europe, however, the situation is complicated by the fact that there is no EU-wide policy of regulation or risk assessment. Across neighbouring member states there are some countries with relatively stringent regulation (e.g. UK, Denmark, Hungary and Sweden) and some without (e.g. France, Greece and Italy) (Loomans, 2007); but of course there is nothing to prevent an organism released in a country without regulation from moving into a country where it would not have been released, as has occurred with the predatory ladybird *Harmonia axyridis* (Pallas) (Coleoptera: Coccinellidae) (Majerus *et al.*, 2006).

The UK, being an island, is in the fortunate position of having considerably more control over the species that arrive in the country. Currently, guidelines derived from the Wildlife and Countryside Act (1981) are relatively stringent, prohibiting the release of non-native species prior to the completion of a comprehensive risk assessment. Under the terms of this

Act, if the assessment is deemed adequate to show minimal risk, exemption licenses can be granted.

1.7 FACTORS AFFECTING THE DEGREE OF RISK TO NON-TARGET ORGANISMS

Howarth (1991) summarised the factors believed to be important in affecting the degree of risk to non-target organisms. These included the permanence of the candidate control agents and their host and habitat ranges.

1.7.1 Permanence

Many organisms released for the purposes of biological control are able to self perpetuate and disperse. This can result in the permanent establishment of a control agent in the alien environment and indeed, in classical biocontrol, these features are essential for the successful control of a pest species. Whilst permanence is often cited as being a desirable characteristic in a control agent, it is potentially very costly to non-target species (Follett *et al*., 2000). The longer an agent persists after introduction, the greater the chance of an increase or shift in both its habitat and host range (Moore, 1987; van Lenteren *et al*., 2003). Therefore, the inundative release of an agent that is unable to establish permanently in an alien environment is likely to pose a lesser threat to the ecosystem (Tiedje *et al*., 1989).

Because of the possible adverse effect to native species, it is essential that a pre-release trial includes a thorough investigation into the establishment potential of a candidate control agent (unless, of course, it is a classical release where establishment is a pre-requisite for success). In order to do this, both biotic and abiotic factors need to be taken into consideration. For example, to assess the ability of a control agent to survive and reproduce in an alien environment, the opportunities for winter survival and availability of non-target species should be investigated (van Lenteren *et al*., 2003). Whilst much of these data will be available in the current literature, a comprehensive report should include assessments of the cold tolerance of the species through the completion of both laboratory and field trials. The need for further tests on the host and habitat range of the control agent is then very much dependent on the outcome of these trials; thus establishment potential often forms the first

part of an ERA (Hart *et al*., 2002a,b; Tullett *et al*., 2004; Hatherly *et al*., 2008; Hughes *et al*., 2009), especially for releases in northern Europe with a distinct winter season.

1.7.2 Host range

Early biological control programmes tended to utilise polyphagous control agents (Simberloff, 1992). At the time this characteristic was considered to be desirable but it is now recognised that such a feature results in an increased risk of attack on non-target species (Howarth, 1985).

If the chosen control agent is strongly host specific, the level of threat is greatly reduced. Furthermore, the ability of such an agent to establish and spread may not be considered detrimental (Howarth, 1991). However, in the event of a control agent having a wider host range, it is important to determine the likely targets. This is not easy. Testing hundreds of potential non-target species would not only be costly and time consuming but, due to the difficulty in accurately predicting the behaviour of the control agent in a novel environment, no method could guarantee absolute safety (Simberloff, 1991). In addition to this there have been concerns about the validity of laboratory examination of host range (Duan & Messing 1996; Van Driesche *et al*., 2003). Duan and Messing (1996) found that the rate of parasitism of a non-target species, lantana gall flies, *Eutreta xanthochaeta* Aldrich (Diptera: Tephritidae), by the fruit fly parasitoid *Diachasmimorpha tryoni* Cameron (Hymenoptera: Braconidae), was 60% in a small laboratory cage and 2% in a large field cage after 24 h, whereas a field release resulted in less than 1% parasitism after one week. Thus, the 'ecological range' of a natural enemy under field conditions is often substantially less than the 'realised' or 'physiological range' assessed in the laboratory (Strand & Obrycki, 1996). In spite of this, in the interest of maintaining the natural dynamics of the ecosystem, this process is essential. A thorough examination of the current literature relating to the host ranges of similar polyphagous insects should enable a suitable range and number of non-target taxa to be selected.

1.7.3 Habitat range

As already stated, there are now several countries that require ERA data for non-native releases, but at present there is no consistency across borders as to what data are necessary. However, insects, being unaware of national boundaries, will invade all suitable habitats within their range (Howarth, 1991). In order to ascertain the level of threat to non-target species as a result of both temporal and spatial encounters, the dispersal ability of the control agent needs to be assessed.

To determine the potential distribution of a candidate control agent several factors, including the life-span of the agent, its mechanism of dispersal and the habitat and climate in the area of release (van Lenteren *et al*., 2003), need to be taken into consideration. van Lenteren *et al*., (2003) stated that if the agent's dispersal ability was limited to less than 10m per season (as in the case of nematodes and fungi), no further information or studies are needed. However, if the agent in question is both able to establish and disperse from the target area, investigations into the rate and range of dispersal and contact with possible alternative hosts are necessary.

1.8 ENVIRONMENTAL RISK ASSESSMENT

Whilst it is widely acknowledged that the introduction of exotic species needs to be regulated for the protection of native ecosystems, there is concern amongst biological control advocates that, as the majority of biological control companies are small with limited research and development budgets, implementation of wide-spread stringent legislation could result in the demise of these so-called environmentally friendly alternatives (Ehlers, 2000). Therefore, for the protection of the environment and the survival of this 'green' alternative to chemical control, it is necessary to strive for a more efficient system of ERA that does not compromise the health of the ecosystem, but at the same time does not impose unnecessary or unaffordable costs on companies that produce beneficial organisms.

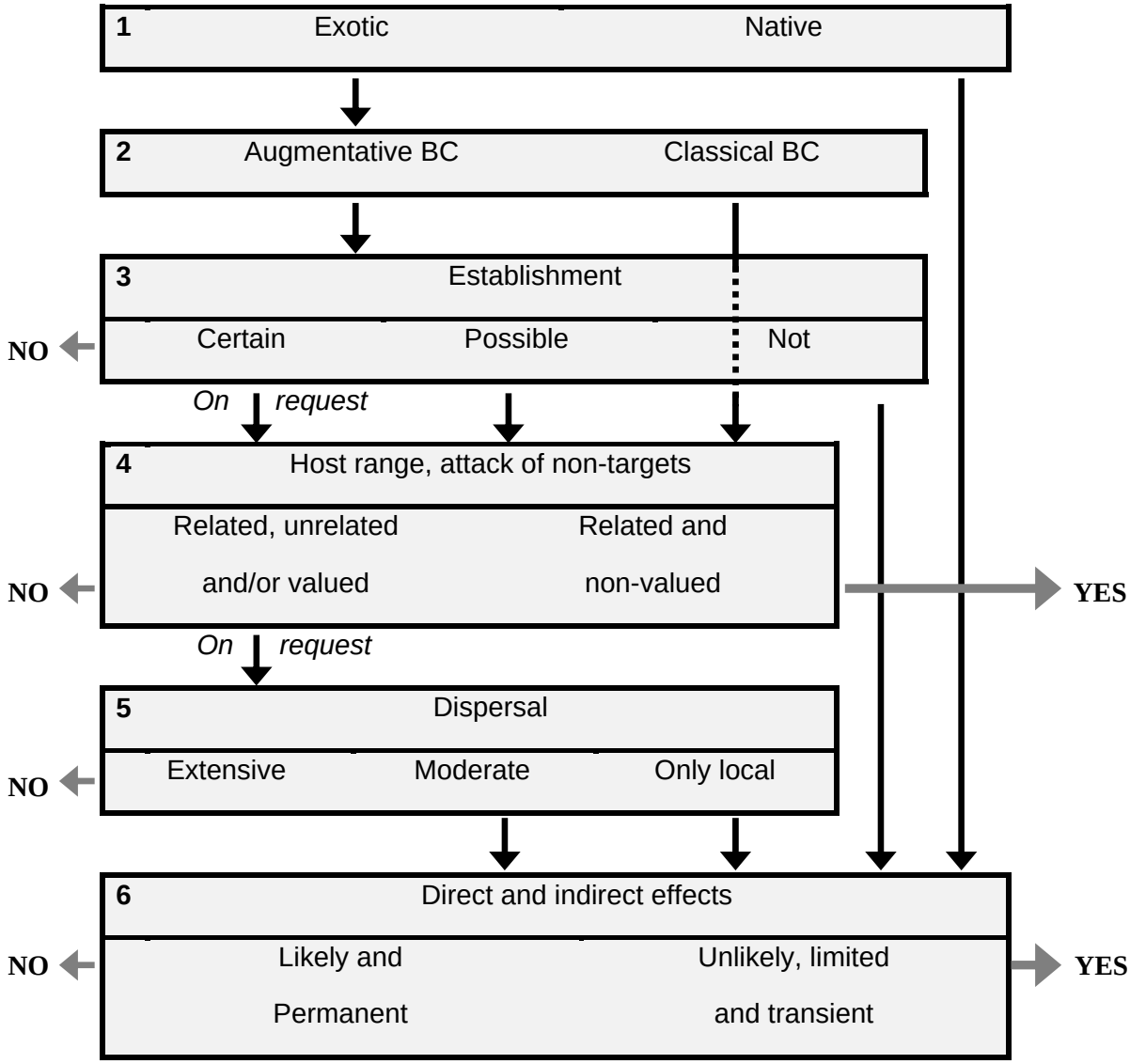


Figure 1.1: Environmental risk assessment scheme for arthropod biological control (BC) agents. NO: release is not recommended; YES: release is recommended (Adapted from van Lenteren *et al.*, 2006).

In 2006, an ERA for invertebrate biological control agents (Fig. 1.1) was devised by van Lenteren & Loomans (2006a,b). This system of risk assessment consists of a stepwise analysis of the above mentioned factors: establishment potential, host range, dispersal abilities and the likelihood of any direct and indirect effects. The development of this hierarchical procedure enables the efficient and cost-effective identification of potentially hazardous agents. This ERA system is also flexible. Thus, if a species is intended for inundative releases within glasshouses in temperate regions, ERA would focus mainly on

cold tolerance. Conversely, for classical or outdoor biocontrol in Mediterranean areas, it is likely that year-round development and reproduction would occur; hence the risk assessment would focus mainly on host range. If it was clear from these investigations that climatic constraints would prevent outdoor establishment, then it would not normally be necessary to investigate other factors such as host or habitat range. In this way, financial resources for the safety-testing of candidate control agents can be allocated in an appropriate way.

1.9 INSECTS AT LOW TEMPERATURES

Temperature is widely regarded as the most important factor affecting the survival and therefore establishment potential of a species in an alien environment (Bale & Walters, 2001). This is especially true of ectotherms due to their limited ability to regulate their body temperature. As a result of this, all insect species are highly dependent on the availability of an adequate thermal budget to allow for both development and reproduction. Without this, establishment in a cooler climate would not be possible. Furthermore, in temperate climates such as the UK, where temperatures are expected to fall below zero on occasions, an insect must be able to tolerate or avoid internal freezing of the body tissue and fluids to survive. Consequently, for releases within glasshouses in temperate regions, the temperature-establishment interaction often forms the first part of an integrated 'environmental risk assessment' for non-native biocontrol agents (van Lenteren *et al*., 2006; van Lenteren & Loomans, 2006a,b).

Factors often investigated to ascertain a species' cold tolerance include the developmental threshold of the organism, its supercooling point (SCP), lower lethal times and temperatures and the organism's ability to overwinter in its intended area of release.

1.9.1 Temperature and development

In the 1770s, the French scientist Reaumur was the first to observe that the developmental rate of an insect is temperature-dependent (Reaumur, 1735). Since this observation, many scientists have investigated the effect of this abiotic factor on the life cycle of exothermic species. Sharpe and De Michele (1977) proposed that temperature limits on growth were as

a consequence of rate-limiting enzymes. Practical analyses have since concurred with this proposal and many models for determining the developmental rates, based on rate-limiting enzymes, have been developed (Wagner *et al*., 1984). However, it has since become clear that enzymes are not the only factor affecting development. Thus no single biologically based model has been developed to account for how temperature alters growth rates (Higley & Haskell, 2001).

To predict the relationship between development and temperature in an insect, a development curve is often used. This curve exists for all invertebrates, although the specifics of the curve will vary between species (Fig. 1.2).

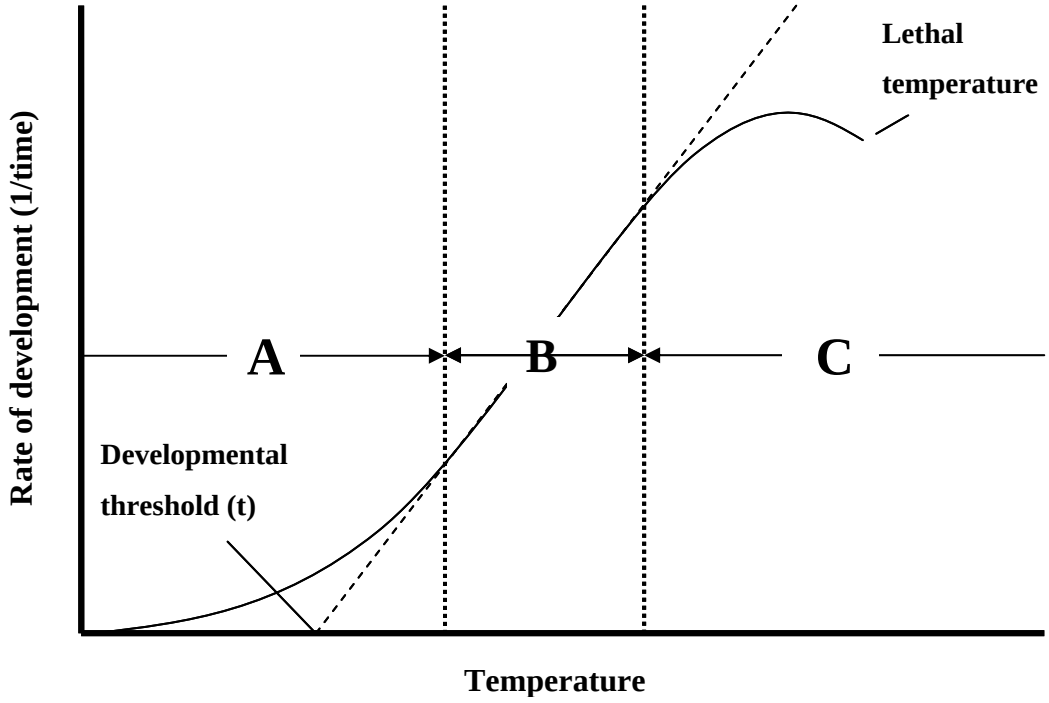


Figure 1.2: Effect of temperature on the rate of development (1/time) of an insect. A and C are non-linear ranges of the development curve, and B is a linear range often used to determine the developmental threshold temperature (t) by extrapolating the line to the x-axis (modified from Campbell *et al*., 1974).

For all insects, development occurs within a relatively narrow range of temperatures (Lamb, 1992). The lower limit of this range, known as the developmental threshold, varies both between species and between different life stages of the same species (Hart *et al*., 1997). As extended exposure to temperatures close to the developmental threshold can be deleterious,

particularly for species of a tropical, sub-tropical or Mediterranean origin, this threshold is often used as a useful indicator of an insect's potential distribution, abundance and establishment potential (Campbell *et al*., 1974).

Using simple linear regression described in Equation 1, it is possible to determine the developmental threshold of a species by extrapolating the linear section of the developmental curve to the x-axis (see Fig. 1.2).

$$\mathbf{R(t) = bT - a}$$

Equation 1.1: Relationship between the rate of development (*R*) and the rearing temperature (*T*) (Lamb, 1992), where *t* is the developmental threshold and *a* and *b* are coefficients of the rectilinear equation.

As temperatures increase, developmental rate reaches a maximum where it levels off and then decreases rapidly to zero as temperatures approach the upper limit. The point at which development ceases at this upper limit is known as the upper developmental threshold. Prolonged exposure to temperatures within this range (zone C) can also lead to mortality (Campbell *et al*., 1974).

1.9.2 Thermal budget and voltinism

The establishment of a species is very much dependent on the organism's ability to develop and reproduce in the area of release. When daily temperatures are below the organism's developmental threshold, no development is possible. At temperatures above this threshold, development increases and day degrees (^{o}d) accumulate. The number of day degrees required for the completion of one generation is measured in Celsius and known as the thermal budget (K). If a full data set of the development of a particular species at a range of temperatures has been obtained, the thermal budget of a species can be estimated using the linear section of the developmental curve by taking the reciprocal of the slope (B) (Equation 1.2). Alternatively, the thermal budget can be calculated from the developmental threshold (t) (Equation 1.3) where R is the rearing temperature and D is the number of days taken to complete development at this temperature.

$$K = 1/b$$

Equation 1.2: Calculation of the thermal budget (Campbell *et al*., 1974).

$$K = D \times (R-t)$$

Equation 1.3: Calculation of the thermal budget from the developmental threshold (Bale & Walters, 2001).

With knowledge of the organism's thermal budget and developmental threshold, coupled with climate data of the intended release site, it is possible to calculate the theoretical number of generations per year. This is known as the voltinism of a species. In the absence of sufficient day degrees for development from egg to adult in a single year, establishment is unlikely (Biovin *et al*., 2006).

1.9.3 Supercooling point (SCP)

The supercooling point of an insect is the temperature at which it spontaneously freezes when cooled below its equilibrium freezing temperature (Wilson *et al*., 2003). The exact point at which this process occurs can be identified by the detection of an exotherm (see Fig. 1.3), the release of the latent heat of fusion that accompanies the process of freezing. Having determined the SCP of an insect, it is then possible to establish if the insect is freeze tolerant or susceptible (Baust & Rojas, 1985).

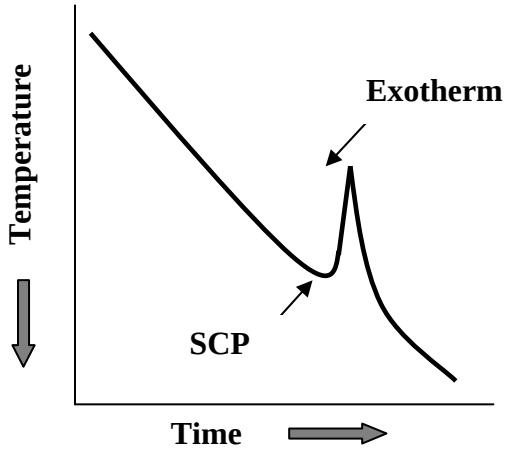


Figure 1.3: Typical cooling and freezing curve of a generalised insect.

In the past, it was believed that the SCP of an insect was a direct measurement of its cold hardiness and, as such, much of the research was concerned with the mechanisms of surviving or avoiding freezing. It is now understood that the greatest threat to survival is cold (Bale, 1987, 1993), as the majority of insects die at temperatures above their SCP. Therefore, whilst the SCP provides accurate information about the lethal temperature of truly freeze avoiding species, and can offer a convenient comparative index (Renault *et al*., 2002), it is essential to avoid placing too much importance on the ecological significance of such measurements alone, especially for species that originate in warm climates.

1.9.4 Lower lethal temperature

As discussed, the majority of insects will die at temperatures considerably higher than their SCP. These lethal temperatures (LTemp) can be determined by exposing insects to a series of sub-zero temperatures, for a set period of time, before re-warming to assess mortality. This LTemp range is often identified as part of an assessment of the establishment potential of an exotic species in temperate regions (Hart *et al*., 2002a,b; Hatherly *et al*., 2003, 2008; Hughes *et al*., 2009), with $LTemp_{50}$ (the temperature resulting in 50% mortality of the population) frequently used as a comparative index of insect cold hardiness.

1.9.5 Lower lethal time

The lower lethal time (LTime) of a species is an index that permits the assessment of chronic exposure to a constant temperature, thus allowing investigation into the survival of an organism over longer time periods at temperatures more likely to be experienced in the field. Again, the exposure time at each temperature resulting in 50% mortality ($LTime_{50}$) is often used as a comparative index of species cold hardiness. Furthermore, through studies of the thermal biology of non-native biological control agents at the University of Birmingham, the survival of invertebrates at 5ºC was identified by Hatherly *et al*. (2005) as a rapid indicator of winter field survival (Fig. 1.4) and was confirmed by subsequent investigations (Hatherly *et al*., 2008; Hughes *et al*., 2009; Allen, 2010). This risk assessment, if proven to be robust, should provide a reliable, quick and effective way of categorising candidate control agents as low, medium or high risk in terms of their ability to establish (see Hatherly *et al*., 2005). The

outcome of this assessment would then determine the need for further tests on the host range and possible non-target effects of the species.

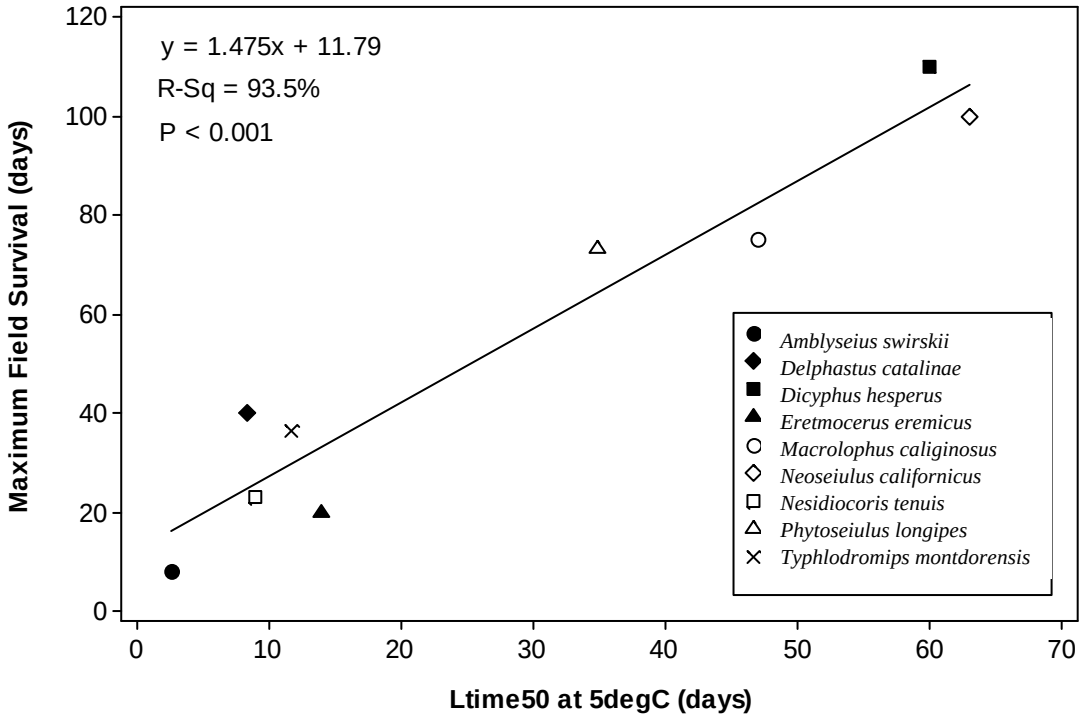


Figure 1.4: Relationship between $LTime_{50}$ at 5°C and the maximum field survival time in winter for non-native invertebrate species in the UK. Sources of data: *Amblyseius swirskii (*Allen, *2010), Delphastus catalinae (*Tullett, 2002), *Dicyphus hesperus* (Hatherly *et al*., 2008), *Eretmocerus eremicus,* (Tullett *et al*., 2004), *Macrolophus caliginosus* (Hart *et al*., 2002b), *Neoseiulus californicus* (Hart, 2002a), *Nesidiocoris tenuis* (Hughes *et al*., 2009), *Phytoseiulus longipes* (Allen, 2010), *Typhlodromips montdorensis* (Hatherly *et al*., 2003).

1.9.6 Rapid cold hardening

The process of rapid cold hardening (RCH) was observed in the flesh fly *Sarcophaga crassipalpis* Macquart (Diptera: Sarcophagidae) (see Chen *et al*., 1987; Lee *et al*., 1987). Up until this point, cold hardening was thought of as being a gradual acclimatory response to the seasonal decrease in temperature and photoperiod approaching winter. However, the work carried out in 1987 showed that a significant increase in cold hardiness could be induced in a matter of minutes or hours. This occurrence is now known to increase cold tolerance in a number of species (Lee *et al*., 2006), including the non cold-hardy house fly, *Musca domestica*. In this species exposure of individuals to 0°C, for as little as 90 min, dramatically increased their survival at -7°C for 2 h from 0% to above 80% (Coulson & Bale, 1990).

For a species that has a single overwintering stage, seasonal (rather than rapid) cold hardening tends to be the dominant response. However, an insect's ability to achieve a rapid increase in cold hardiness is useful in spring and autumn where sudden, unpredictable decreases in temperature may occur when the species is not yet fully adapted to such extremes (Coulson & Bale, 1990). This rapid response is also thought to be especially important in species such as aphids, where a seasonal response may not be possible due to the species' short generation time (Powell & Bale, 2004).

1.10 CLASSES OF COLD TOLERANCE

Some species, such as the high arctic moth, *Gynaephora groenlandica* (Lepidoptera: Lymantriidae) possess the remarkable ability to survive temperatures as low as -30 to -70°C (Kukal, 1991). Whilst the majority of species are never exposed to such severe conditions, all insects have been broadly classified into one of two main categories of cold hardiness: freeze tolerance and freeze avoidance.

Whilst these categories are still in use today, in light of the discovery of extensive pre-freeze mortality in many species (Knight *et al*., 1986; Bale, 1991), Bale (1993) proposed a re-classification of strategies of cold tolerance into five groups: freeze tolerant, freeze avoiding, chill tolerant, chill susceptible and opportunistic survival. These categories have now been further divided to encompass the different levels of cold hardiness observed within the freeze tolerant group (Sinclair, 1999).

1.10.1 Freeze tolerant insects

Through the production of various biochemical compounds, freeze tolerant insects have the ability to survive the internal formation of ice (Salt, 1961), a process that can be lethal in the absence of necessary adaptations. Three of these substances, antifreeze proteins (AFPs) and cryoprotective polyols and sugars, are also utilised by freeze avoiding species. The major distinction between the two strategies is the occurrence of ice nucleating agents (INAs) in freeze tolerant species to initiate protective extra-cellular freezing at elevated temperatures.

In contrast, freeze avoiding species must remove or mask such compounds to allow the necessary level of supercooling.

The freeze tolerant category has been sub-divided to encompass the different levels of freeze tolerance seen within the group (see Sinclair, 1999). Insects within these categories, partially freeze tolerant, moderately freeze tolerant, strongly freeze tolerant and freeze tolerant with a low SCP, are all able to tolerate freezing but differ in the extent to which they can survive a reduction in temperature to levels near or below their SCP. The definitions given by Sinclair (1999) are as follows:

- Partially freeze tolerant insects are able to survive the formation of some ice in the body, but do not survive if ice formation goes to an equilibrium (or above) the SCP.
- Moderately freeze tolerant species freeze at relatively high temperatures, and die at temperatures less than 10^{o}C below their SCP.
- Strongly freeze tolerant insects are able to survive temperatures significantly below that of their SCP.
- Freeze tolerant with a low SCP species are able to survive temperatures just below their SCP. However, due to their low SCP, these temperatures can be as low as -55^{o}C, as in the case of the beetle *Pytho deplanatus* (Coleoptera: Pythidae) (Ring, 1982).

1.10.2 Freeze avoiding insects

Very few species are able to tolerate freezing and few die from freezing alone (Somme, 1982; Bale, 2002). The majority of insects will therefore endure the cold and resist the internal formation of ice through the ability to supercool. This is achieved through a variety of behavioural and physiological mechanisms and by the increase in the synthesis of polyols and AFPs. These processes enable an insect to lower their SCP significantly, thereby avoiding freezing until relatively low sub-zero temperatures are experienced. An example of such a species is the autumnal moth, *Epirrita autunma* (Borkhausen) (Lepidoptera: Geometridae). This species is one of a relatively small group of insects that can be accurately described as

freeze avoiding, in other words, a species in which little or no mortality occurs in the absence of freezing (Bale, 2002).

1.10.3 Chill tolerant insects

The chill tolerant group encompasses a large number of species that are able to survive low or sub-zero temperatures but show some pre-freeze mortality at temperatures above their SCP (Bale, 1993). This group can also be further sub-divided into highly and moderately chill tolerant insects, allowing for a more accurate description of the cold tolerance of species across a range of climatic zones.

The examples given by Bale (1993) to exemplify these sub-groups are the highly chill tolerant Antarctic mite, *Alaskozetes antarcticus* Michael (Acari: Cryptostigmata) (see Cannon & Block, 1988), and the beech weevil, *Rhynchaenus fagi* L. (Coleoptera: Curculionidae), a moderately chill tolerant species. *A. antarcticus* is able to tolerate sustained exposure to sub-zero temperatures of -5 to -15°C (Davey *et al.*, 1992). This high level of cold hardiness, accompanied by the buffering effect of snow, enables the species to survive the harsh Antarctic winters (Davey *et al*., 1992). Whilst it is clear that this species is indeed very cold hardy, it would be inaccurate to describe it as completely freeze avoiding, as a level of mortality has been recorded at temperatures above its SCP (see Cannon, 1987). *R. fagi* is considered to be less cold hardy due to the relatively high mortality of the population at temperatures significantly above the SCP. When exposed to -15°C for 50 d (10°C above the SCP), less than 30% of the population survive (Bale, 1991). So, whilst the cold hardiness of this weevil is more than adequate for the milder winter temperatures experienced in the climate in which it lives, it is evident that in comparison to species such as *A. antarcticus*, it can only be viewed as moderately chill tolerant.

1.10.4 Chill susceptible insects

Chill susceptible species are able to survive temperatures below their developmental thresholds, but die rapidly after exposure to relatively high sub-zero temperatures (Bale, 1993). An example of such a group is the aphids. Although these insects tend to have an

overwintering SCP of approximately -25°C, 100% mortality is seen following exposure for 1 min to temperatures between -5° and -15°C (Bale *et al.*, 1988).

1.10.5 Opportunistic survival

The final group, the opportunistic survivors, are distinguished from the other groups by their inability to endure temperatures below their developmental threshold (Bale, 1993). These species are often tropical or sub-tropical in origin and, as such, are unable to reduce their metabolism to aid survival (Bale, 1991). An example of such an insect is the *M. domestica*. Coulson and Bale (1990) found that 90% of pupae died after 4 d exposure to 0°C and Somme (1961) showed that at 5°C, 97.5% of the larvae died after 8 d. As a result of these investigations, it has been concluded that the winter survival of *M. domestica* is dependent on the species' ability to exploit suitable warm habitats and sites opportunistically.

1.11 BIOCHEMISTRY OF LOW-TEMPERATURE SURVIVAL

Both freeze tolerant and freeze avoiding insects synthesise a variety of biochemical compounds to achieve a winter cold hardy state. Antifreeze proteins and cryoprotective compounds are produced by both categories of insects to depress the SCP relative to the melting point. In addition to these, freeze tolerant insects will also produce INAs to encourage the formation of ice at elevated sub-zero temperatures.

1.11.1 AFPs

AFPs, also known as thermal hysteresis proteins, are thought to play an important role in the protection of both freeze avoiding and freeze tolerant insects at low temperatures, although their role in freeze tolerant insects remained elusive for a number of years. These proteins lower the freezing point of water without significantly affecting the melting point (Duman, 2001). In freeze avoiding species, this increase in supercooling capacity protects the organisms by enabling them to avoid the internal formation of ice at relatively low sub-zero temperatures, whereas in freeze tolerant species the proteins function as antifreeze agents in

spring and autumn where low levels of INAs leave the insects vulnerable to the formation of intracellular ice. In addition to this, AFPs have a cryoprotective role in spring, acting to prevent secondary re-crystallisation of internal ice, a process associated with the increase in temperature towards the end of the winter (Duman, 2001).

1.11.2 INAs

INAs are produced exclusively in freeze tolerant insects. They are usually synthesised in autumn or early winter to encourage the formation of ice crystals in extracellular spaces at relatively high temperatures of -10°C or above (Bale, 2002). The formation of ice in these areas prompts the movement of water out of cells into these spaces to restore osmotic equilibrium. The resulting desiccation of cells increases the concentration of cellular solutes, thereby reducing the intracellular freezing temperature and thus decreasing the risk of freezing.

In general, these compounds are lost in the spring. Therefore, during this period, the insects have to rely on the ability to supercool for survival. However, in areas where freezing is possible throughout the year some species retain these compounds, as in the case of the adult beetle *Phyllodecta laticollis* (Coleoptera: Chrysomelidae) in Norway (van der Laak, 1982), though the level of freeze tolerance is less in summer.

1.11.3 Cryoprotective compounds

Cryoprotective compounds such as polyhydroxy alcohols (polyols) and sugars are, like AFPs, produced in both freeze tolerant and freeze avoiding insects. These compounds, of which glycerol and sorbitol are the most common and abundant (Somme, 1964; Baust, 1973; Zachariassen, 1985), tend to be produced in late autumn and are absent or occur at low levels during summer.

Free glycerol was first identified in the haemolymph of diapausing pupae of the silk moth, *Hyalophora cecropia* (L.) (Lepidoptera: Saturniidae) (Wyatt & Kalf, 1956) and Chino (1957) found that glycogen was converted into glycerol and sorbitol in the eggs of *Bombyx mori* L. (Lepidoptera : Bombycidae) on entry into a diapause state, and re-synthesised upon diapause termination. As glycerol had long been recognised as a cryoprotective compound (Smith,

1950), these discoveries greatly enhanced the understanding of the mechanisms by which invertebrates tolerate the cold and freezing. Polyols and sugars are now known to counteract the damaging effects of low temperatures in a number of ways, including the reduction in the rate of ice crystal growth, stabilisation of protein structure, reduction in transmembrane water fluxes and maintenance of cell volumes above the critical threshold (Baust, 1973, 1982).

1.12 BEHAVIOURAL AND PHYSIOLOGICAL CHANGES

As well as producing a number of biochemical products to achieve a cold hardy state, many species will also employ a variety of behavioural and physiological mechanisms. These include, amongst others, the location of an overwintering site, the cessation of feeding and evacuation of the gut (Bale, 2002).

The location of a sheltered overwintering position is an important component of the survival of many overwintering species (Danks, 1991, 2006). For example, species that overwinter in sites that accumulate deep snow are afforded significant insulation from the lower air temperatures. An example of an insect that utilises this method of winter survival is the aforementioned mite, *A. antarcticus*. Whilst this species shows considerable cold hardiness with the ability to tolerate sustained exposure to temperatures as low as -15°C, it inhabits one of the coldest places in the world where air temperatures can decrease to -40°C. Thus, without the ability to select an appropriate snow-covered microhabitat, this species would be much less likely to survive the winter.

An additional benefit to selection of a sheltered overwintering site is the reduction in the rate of temperature change at such a site (Danks, 2006). This is often critical to the survival of a species as many insects with the ability to survive freezing are able to do so only when the rate of cooling is low (Miller, 1978; Bale *et al.*, 1989). For freeze avoiding species, the ability to survive low temperatures above their SCP is also influenced in this way by the rate of cooling (Coulson & Bale, 1990; Kelty & Lee, 1999). This increase in survival is probably due to the additional time available for the production of biochemical components and the onset of various behavioural and physiological changes, vital for the survival of low and sub-zero temperatures.

In freeze intolerant insects, the organism must avoid freezing to survive. One way in which this is often achieved is through the removal of INAs from the gut through the cessation of feeding and gut evacuation (Somme & Block, 1982; Cannon & Block, 1988), measures that have been shown to depress the SCP of a number of species (e.g. Salt, 1961; Krunic, 1971; Block, 1990; Gehrken, 1995). In fact, it has been established that in some species, these changes alone are sufficient to decrease the SCP to -20°C (Leather *et al.*, 1993).

1.13 DIAPAUSE

Diapause is a form of dormancy that is widespread amongst insects and related arthropods (Tauber & Tauber, 1976; Denlinger, 2002). This dormant period enables a species to tolerate adverse conditions and is therefore often an essential prerequisite for successful overwintering (Denlinger, 1991; Slachta *et al.*, 2002). Furthermore, diapause is thought to play an important role in the synchronisation of vital activities such as growth and reproduction, ensuring that they occur during favourable periods of the year (Tauber & Tauber, 1976).

1.13.1 Diapause induction

Diapause is broadly divided into two types; obligatory and facultative. Obligatory diapause often occurs in univoltine insects to extend their short life-cycle to one year (Gullan and Cranston, 2010). This form of diapause occurs at a fixed time regardless of prevailing environmental conditions. However, facultative diapause is optional and is initiated by environmental cues, the primary cue typically being the reduction in day length in late summer and early autumn (Tauber *et al*., 1986). This environmental trigger enables the organism to anticipate the arrival of winter in advance of the onset of low temperatures, thereby allowing sufficient time for the selection of a suitable overwintering site and the accumulation of energy reserves (Denlinger, 2002). In many species capable of entering a diapause state in winter there exists a critical day length below which the majority of the population will enter diapause (Tauber & Tauber, 1981). However, it is known that in certain species temperature is able to affect the timing of diapause by altering the critical photoperiod (Danileveskii, 1965; Tauber *et al*., 1986) and in other cases temperature induces diapause

independently of photoperiod (Veerman, 1992). Other environmental factors known to play a role in the initiation of diapause include resource availability, temperature and relative humidity. However, these factors are thought to be less important as they do not provide a reliable indication of seasonal change.

Diapause is characterised by a number of behavioural, physiological and biochemical features and, in many cases, it is possible to diagnose the diapause condition by analysing the physical form of a species (Tauber & Tauber, 1976). For example, in phytoseiid mites, the diapausing females tend to be flatter and less active (Hoy & Flaherty, 1970: Croft, 1971; Overmeer, 1985) and in the silkworm, *B. mori*, identification of diapausing eggs is possible through the analysis of yolk granule distribution (Takesue, 1976). However, the most common and accurate criterion used to identify the diapause condition is an inability of the females to develop eggs and oviposit (e.g. Overmeer, 1985).

1.13.2 Diapause and Cold Hardiness

It is evident that the capacity to arrest development in order to endure harsh conditions has contributed greatly to the success of the Class Insecta, enabling them to exploit a wide variety of habitats from the tropics to the poles (Denlinger, 2002).

In some insects, the ability to diapause is essential for successful overwintering (Denlinger, 1991; Slachta *et al.*, 2002), although many species are able to attain a significant degree of cold hardiness in the absence of this trait (Fields *et al.*, 1998). However, as a number of studies have shown that the acclimatory ability of diapausing individuals is greater than that of non-diapausing individuals, it is important to investigate the capacity of a candidate control agent to enter this dormant state as it may aid its ability to establish outside the glasshouse.

1.14 BEHAVIOUR AT LOW TEMPERATURES

As discussed, the range of temperatures over which insects and mites can survive is limited by upper and lower lethal thresholds. However, within this range lies a more restricted zone within which insects are capable of normal locomotory function (Mellanby, 1939). Whilst

organisms are capable of survival at temperatures above and below those which result in immobility, sustained exposure to such conditions would invariably lead to mortality as a result of an inability to find food resources, avoid predation or breed successfully. Therefore, in temperate regions where conditions are rarely severe enough to result in direct mortality, measures of these movement thresholds may provide a more ecologically relevant indication of establishment potential.

Traits often examined include non-lethal measures such as chill coma temperature (Mellanby, 1939; Colhoun, 1960; Gaston & Chown, 1999), chill coma recovery (Ayrinhac *et al*., 2004; Casteneda *et al*., 2005; Macdonald *et al*., 2004) and motility across a range of temperatures (Allen, 2010, Hughes *et al*., 2010).

1.14.1 Chill coma

In temperate climates ectotherms have to endure cold stress to survive. Chill coma is one way in which they are able to do this.

The first observable physiological response to cooling is a decrease in walking speed. When cooling is continued, a temperature is reached at which the organism loses locomotory function; this is the insect's critical thermal minimum (CT_{min}) (Cowles & Bogert, 1944). A further reduction in temperature renders the organism completely immobile, a state known as chill coma. Providing this cold treatment is not too prolonged, this narcotic state is reversible (Gibert *et al*., 2001). However, whilst the organisms are in this state, they are unable to find food or avoid predation (Kelty & Lee 1999). Thus, exposure to temperatures which impede movement can indirectly cause death.

The temperature at which this narcotic state is observed is often used as an estimate of the cold tolerance of a species (Schenker, 1984; Leather *et al*., 1993; Convey, 1997).

1.14.2 Chill coma recovery

In temperate climates where diurnal and seasonal temperature fluctuations often result in the exposure of ectothermic species to deleterious conditions, the ability to tolerate and recover rapidly from cold exposure is a fitness advantage (Gibert *et al*., 2001). In accordance with

this, the temperature at which an organism regains locomotory function following chill coma has often been cited as a good measure of cold tolerance in arthropod species (see Parsons, 1983; Gilbert *et al*., 2001; David *et al*., 2003; Ayrinhac *et al*., 2004; Castaneda *et al*., 2005). This indicator of cold tolerance is known as the 'chill coma recovery' temperature.

The validity of chill coma recovery as a measure of cold tolerance has been supported by observations that at higher latitudes, or areas with lower annual mean temperatures, recovery time from chill coma is shorter (Gilbert *et al*., 2001; David *et al*., 2003; Ayrinhac *et al*., 2004; Castaneda *et al*., 2005).

1.14.3 Walking speed

Insects are cold blooded organisms and, as such, their activity is very much dependent on the ambient temperature. Therefore, when an insect or mite is cooled, one of the first observable physiological responses is a decrease in walking speed.

Whilst studies often focus on the temperature at which movement ceases, from a biological control perspective, it is also interesting to measure the speed of movement at temperatures where coordinated locomotory function is possible. For example, when assessing the efficacy of a control agent, it would seem advantageous for the predator or parasitoid to be more active than the pest species across a range of temperatures. In accordance with this, searching speed is often adopted as a measure of agent quality (Bigler, 1989; Cerutti & Bigler, 1995; Hughes *et al*., 2010).

1.15 *NESIDIOCORIS TENUIS*

Mirids have traditionally been thought of as phytophagous agricultural pests, causing damage to crops such as cotton (*Gossypium* spp.) (Wu *et al.*, 2002; Fitt *et al.*, 2004; Urbaneja *et al.*, 2005) and tobacco (*Nicotiana tabacum* L.) (Kessler & Baldwin, 2004). Recently, however, mirids have received recognition for being polyphagous insects that could potentially be useful as biological control agents (Gabarra, 1995; Albajes & Alomar, 1999; Alomar *et al*., 2002; Lucas & Alomar, 2002; Urbaneja *et al.*, 2005).

One such species, *Nesidiocoris tenuis* Reuter (Hemiptera: Miridae) is a species of mirid of Mediterranean origin which has been shown to make a significant natural contribution to the control of pests such as whitefly (*Bemisia argentifolii*), leafminers (*Liriomyza* spp.), thrips (*Thrips* spp.) and spider mites (*Oligonychus* spp.) within that region (Ryckewaert & Alauzet, 2002; Urbaneja *et al*., 2005). Whilst *N. tenuis* is a zoophytophagous species which can feed on certain host plants (e.g. tomato (*Solanum lycopersicum* L.)), causing feeding punctures leading to possible infection and flower abortion when prey is scarce or absent (Vacante & Garzia, 1994; Calvo & Urbaneja, 2003), research has shown that the financial gain as a result of reduction in pest numbers greatly exceeds any losses (Vacante & Garzia, 1994).

As a result of these observations, *N. tenuis* is now mass reared and supplied to areas in south-eastern Spain and the Canary Islands for use as a biological control agent on crops such as tomato (Urbaneja *et al.*, 2005). As this mirid has proven to be a valuable control against important horticultural and ornamental pests, it may have further applications in glasshouse biological control within northern Europe, especially if there was no likelihood of establishment in the wider environment.

1.15.1 Life cycle of Nesidiocoris tenuis

The life cycle of *N. tenuis* is comprised of an egg stage, five nymphal instars and adult (Fig. 1.5). Nymphal instars 1 and 2 are very similar in appearance but can be differentiated by size or the presence of discarded exuvia. Instars 3, 4 and 5 are easier to identify due to the formation of ‘wing buds’ (see Fig. 1.5). Only the adults possess fully functional wings and are therefore the only stage with the capacity to fly. One of the benefits of *N. tenuis* as a control agent relates to the fact that all stages, except the egg, are active and able to feed and thus control of the pest species can be achieved continuously.

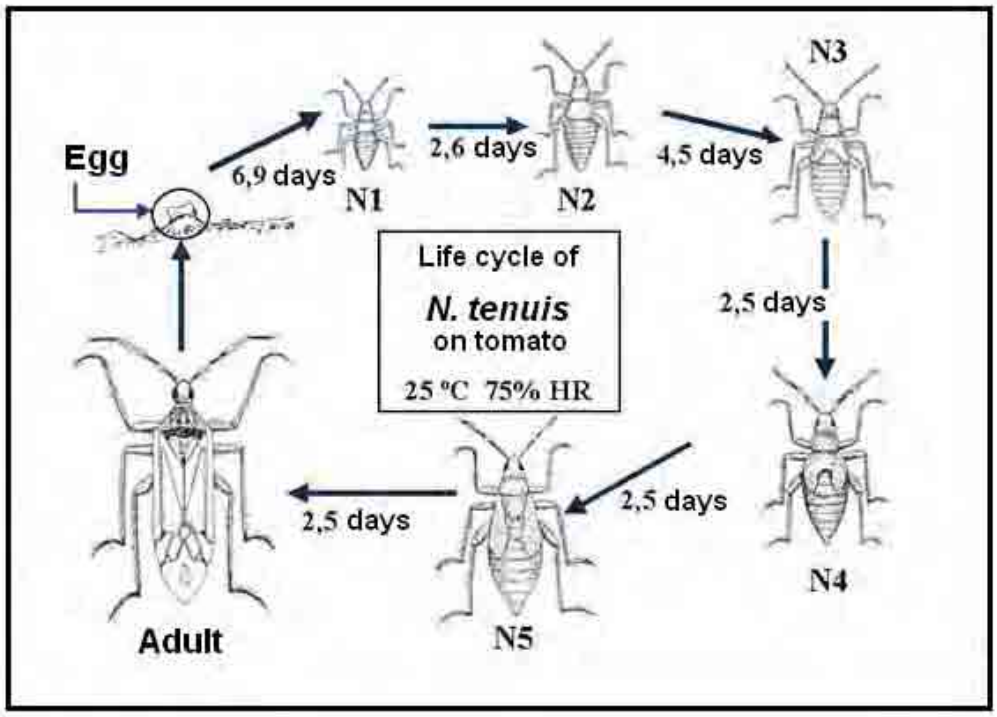


Figure 1.5: Life cycle of *Nesidiocoris tenuis* on tomato at 25°C, 75% relative humidity. Diagram adapted from Calvo and Urbaneja (2003).

1.15.2 Host plant and prey effect on development and survival

N. tenuis is capable of development on a range of plant species including tomato (*S. lycopersicum*), pepper (*Capsicum annuum L.*), aubergine (*Solanum melongena*), cucumber (*Cucumis sativus* L.) and beans (*Fabaceae* spp.) (Calvo & Urbaneja, 2004). However, unlike mirids such as *D. tamaninii* Wagner (Heteroptera: Miridae), *Macrolophus costalis* Fieber (Heteroptera: Miridae) and *Macrolophus pygmaeus* Rambur (Hemiptera: Miridae), it is unable to complete development to adulthood on a strictly phytophagous diet (Urbaneja *et al*., 2005). Thus the availability of prey is essential for the establishment of this mirid on agricultural crops (Margaritopoulos *et al.*, 2003).

Although *N. tenuis* is unable to complete its lifecycle on plant material alone, it has been noted that the survival and life cycle duration of the mirid in the absence of prey are strongly influenced by the species of host plant (Margaritopoulos *et al.*, 2003; Calvo & Urbaneja 2004), with survival being significantly higher and developmental time shorter on hairy

leaved plants such as aubergine and tomato, rather than pepper (Calvo & Urbaneja, 2004). These findings explain the observed variation in the extent to which different crops are utilised as a food source (see Gabarra *et al.*, 1988) and suggest that, due to the low tolerance for feeding punctures in glasshouse crops, the use of these mirids as control agents may be more feasible on certain plant species than others. These data also imply that *N. tenuis* should only be applied when pest densities are relatively high. Accordingly, field observations have shown that the damage to tomato plants is greatest when *N. tenuis* populations exceed those of the pest species (Trottin-Caudal & Millot, 1997; Calvo & Urbaneja, 2003). Although economic thresholds are rarely reached during these occasions (Vacante & Garzia, 1994), some farmers have reportedly resorted to applying an insect growth regulator (lufenuron) in order to reduce numbers below damaging levels (Monserrat, pers. comm. in Urbaneja *et al*., 2005). Whilst this highlights a major drawback to the use of these mirids, *N. tenuis* has proven to be a valuable tool against important agricultural pests and, as such, there is now the idea of supplying them to other countries and into other types of agricultural ecosystems, such as glasshouses.

1.16 *LYSIPHLEBUS TESTACEIPES*

Parasitoids have long been known to have a major impact on natural and agricultural ecosystems where they regulate the population density of their hosts (Godfray, 1994). As a result of their natural propensity to control pest species, parasitoids have been reared for augmentative release within glasshouses for a number of years. In fact, as discussed, the parasitoid *E. formosa* was the first species to be introduced into glasshouses as a biological control agent. As a result of such successes in these early schemes, insect parasitoids are now the most common organisms used for the classical control of arthropod pests (Parry, 2009).

Lysiphlebus testaceipes (Cresson) (Hymenoptera: Braconidae, Aphidiinae) is an important biological control agent for a broad range of aphid species (Starý *et al*., 1988a,b; Starý, 2004; Silva *et al*., 2008), including the economically important pest of cereal crops, *Schizaphis graminum* (Rondani) (Jackson *et al*., 1970; Rodrigues & Bueno, 2001). This parasitoid, originally introduced to southern France from Cuba in the 1970s, spread rapidly over Mediterranean France, Italy and Spain (Starý *et al*., 1988, 2009). The parasitoid was

originally on the EPPO (European and Mediterranean Plant Protection Organisation) 'positive list' of so called 'safe species' (List of biological control agents widely used in the EPPO region), but was removed from the list in 2008 by the EPPO-IOBC (International Organisation for Biological and Integrated Control of Noxious Animals and Plants – Western Palaearctic Regional Service) joint panel because of concern about its wide and apparently increasing host and habitat ranges (Mackauer & Starý, 1967; Ippolito & Parenzan, 1982; Starý *et al.*, 2004) and evidence that it had become the dominant parasitoid in some areas (Tremblay, 1984; Starý *et al.*, 2004, 2009), displacing native species (Tremblay, 1984). However, as *L. testaceipes* has never been released in the UK, and other countries in northern Europe, it may have potential as a glasshouse biocontrol agent in these regions. Such use would though depend on evidence that the parasitoid was unable to establish populations outside of glasshouses.

1.16.1 Life cycle of Lysiphlebus testaceipes

Lysiphlebus testaceipes is a small, black parasitoid of the sub-family Aphidiinae, a group of species that exclusively parasitize aphids. The life cycle of *L. testaceipes* is comprised of an egg, four larval instars and a pupal and adult stage (Fig. 1.6). The egg, larval and pupal stages of the parasitoid life cycle occur within an aphid host. Adult parasitoids mate and the females oviposit within the abdomen of a suitable host. Within a certain period of time (dependent on ambient conditions), the larva hatches from the egg and begins to feed internally on the living aphid. The movement and growth of the larva within the aphid gives its host a swollen appearance. Eventually the larva kills the aphid, cuts a hole in the bottom of the host exoskeleton and produces a substance that 'glues' the body of the aphid to the leaf. By this point, the aphid has become tanned and hardened, forming a protective shell, or 'mummy' around the developing parasitoid. The larva then pupates, and the adult parasitoid emerges from a circular hole cut in the top of the mummy. Parasitoids tend to overwinter inside aphids, either as larvae within living hosts, or as pupae within mummified aphids.

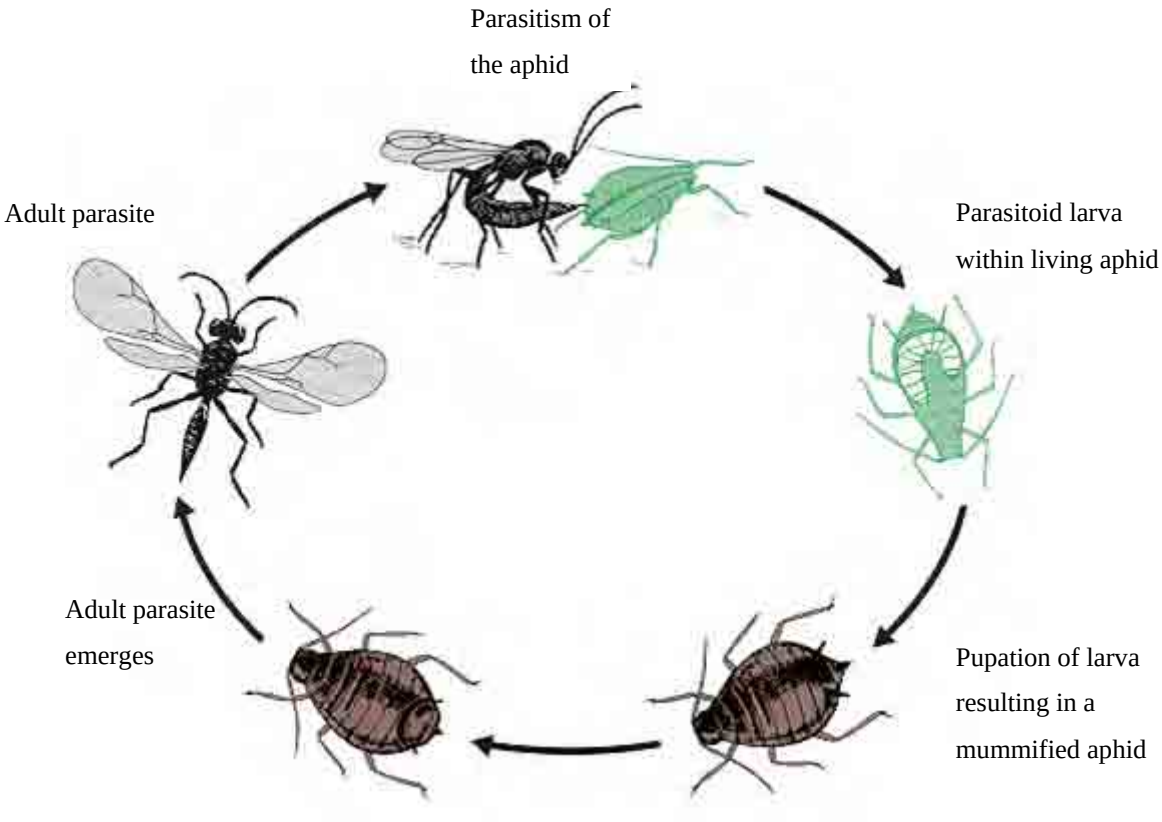


Figure 1.6: Life cycle of aphid parasitoid *Lysiphlebus testaceipes*. Diagram adapted from: insects.tamu.edu/extension/bulletins.

1.16.2 Efficacy of Lysiphlebus testaceipes

Unlike *N. tenuis*, *L. testaceipes* does have an inactive stage; therefore the efficacy of the parasitoid is very much dependent on ambient conditions, as low temperatures would increase the duration of the pupal stage. As many aphids are able to overwinter as active life stages, it is possible that aphid numbers could remain unchecked during the colder months. However, the fecundity of *L. testaceipes* females under suitable conditions is high. One study calculated that the average life-time fecundity of female *L. testaceipes* at 20 and 25ºC was 128.2 and 180 eggs, respectively (van Steenis, 1994). In addition to this, as well as directly controlling aphid population size through parasitism, studies have shown that parasitoids are able to further contribute to the regulation of these pest species by reducing their reproductive potential and increasing their mortality and dispersal through the harassment of the population (Tamaki *et al*., 1970; Ruth *et al*., 1975).

1.16.3 Host selection

Many studies have shown that parasitoids respond strongly to plant volatiles, particularly when the leaves are infested with their host species (Grasswitz & Paine, 1993; Reed *et al*., 1995). However, reports for *L. testaceipes* are conflicting, with some studies finding this to be the case (Grasswitz & Paine, 1993) and others observing that *L. testaceipes* does not appear to have a preference and is equally attracted to uninfested plants (Pinto *et al*., 2004).

Once *L. testaceipes* has located an infested plant, aphids are detected through the apparently random searching of leaves followed by antennal contact. The parasitoid then ascertains the suitability of a potential host through antennal and ovipositor probing (Rodrigues & Bueno, 2001) and decisions are made based on the ability of the host to satisfy the minimal physiological and dietary needs for the development and growth of the immature insects (Mackauer *et al*., 1996). Whilst sexual dimorphism in body size is common in parasitoids, the size of both males and females are very much constrained by host quality, namely the resources available to the parasitoid throughout development (Mackauer & Sequeira, 1993).

1.17 OBJECTIVES

The main aim of this project is to determine the establishment potential of two candidate control agents, *N. tenuis* and *L. testaceipes*, in the UK. As temperature is the most important factor affecting the establishment of alien species in temperate regions (Bale & Walters, 2001), the overwintering potential of the invertebrates will be investigated through a series of laboratory and field experiments. If it is deemed possible for the organisms to tolerate winter conditions, the ability of the insects to locate and utilise wild prey will be assessed to determine the possibility of long-term establishment.

The results of these studies will then be compared with data compiled through analysis of other candidate control agents to examine the hypothesis that laboratory indices of cold tolerance, such as the $LTime_{50}$ at 5ºC, and the maximum field survival time, are correlated (see Hatherly *et al*., 2005). It is hoped that, if these data support the correlation, the $LTime_{50}$ could provide a reliable, quick and easy indication of the establishment potential of alien biological control agents in temperate climates such as the UK (see Hatherly *et al.*, 2005).

In addition to this, a novel technique described by Hazell *et al*. (2008) will be used to investigate various behavioural and physiological thresholds in the candidate control agents. These data will allow further inferences to be made regarding the establishment potential of the species, and will also provide an indication of efficacy of the control agents under a range of conditions.

CHAPTER 2

General Materials and Methods

2.1 INTRODUCTION

In this chapter, the culturing techniques for rearing populations of *Nesidiocoris tenuis* Reuter (Hemiptera: Miridae) and *Lysiphlebus testaceipes* (Cresson) (Hymenoptera: Braconidae, Aphidiinae) are described, as are the general methods used to quantify laboratory and field indices of cold tolerance. Details specific to individual species are given in chapters 3 to 6.

2.2 CULTURING INSECT POPULATIONS

2.2.1 Rearing of Nesidiocoris tenuis

A sample of *N. tenuis* was supplied by Biobest, NV (Westerlo, Belgium) from a population originally sourced from Morocco. The mirids were reared under quarantine conditions at 23°C, 16:8 LD on *N. tabacum* plants and their diet was supplemented with eggs of *Ephestia kuehniella* (Zeller) (Lepidoptera: Pyralidae). This culture was allowed to complete at least two generations under these conditions prior to use in laboratory or field experiments.

To obtain nymphs for laboratory or field trials, 50 adult mirids were placed on young tobacco plants within secure ventilated containers for 24 h, to allow time for mating and oviposition, after which all of the insects were removed. The emerging nymphs were then used following moult to second or third instar, or placed at 10°C for 7 d to acclimate prior to use in field or laboratory experiments. When adult mirids were required, fifth instar nymphs were placed on fresh tobacco plants with a supply of *Ephestia* eggs. Mirids that were synchronised to moult within 48 h of one another were used for all experiments. Acclimated adults were obtained by placing newly moulted individuals at 10°C for 7 d. Acclimated treatments were

maintained for the duration of the acclimatory period on fresh tobacco plants with *Ephestia* eggs as a supplementary food source.

2.2.2 Rearing of Tetranychus urticae

Tetranychus urticae Koch (Acari: Tetranychidae), supplied by Biobest, NV (Westerlo, Belgium), were reared on dwarf French beans, *Phaseolus vulgaris* L. (Fabaceae) at 23°C, 16:8 LD. Bean plants were grown in a separate growth room (20°C, 16:8 LD) and 16 plants of 20 cm height were added to the *T. urticae* culture at weekly intervals. Heavily infested leaves from old plants were removed and transferred onto younger plants to maintain a healthy stock of *T. urticae*. Adult *T. urticae* were used for all experiments and acclimated samples were obtained by placing the adults at 10°C for 7 d.

2.2.3 Rearing of Lysiphlebus testaceipes

L. testaceipes were supplied by Biobest, NV (Westerlo, Belgium). The parasitoids were reared under quarantine conditions at 23°C, 16:8 LD on *Vicia faba* L. plants infested with *Aphis fabae* Scop. (Hemiptera: Aphididae), also supplied by Biobest. The culture was allowed to complete at least two generations under these conditions prior to use in laboratory or field experiments.

To obtain larvae within parasitized aphids, six parasitoids were placed in each of 50 Blackman boxes (Blackman, 1971) containing *V. faba* leaves infested with 10 second or third instar aphids. To allow time for mating and oviposition, the parasitoids were left in the Blackman boxes for 4 h. Following removal of the parasitoids, the aphids were maintained at 23°C for 4 d to ensure that, within the successfully parasitized hosts, the eggs had hatched and larvae had emerged (preliminary investigations identified this period by dissection of aphids at daily intervals following oviposition). The parasitized aphids were then used directly as desired, or maintained in Blackman boxes on fresh *V. faba* leaves at 10ºC for 7 d to acclimate prior to use in field or laboratory experiments. When pupae were required, parasitoids were released into BugDorms (BD2120, BugDorm.megaview.com.tw) containing aphid-infested *V. faba* plants. Mummies that formed within 24 h of one another were used

for all experiments. Acclimated pupae were obtained by placing the newly formed mummies at 10°C for 7 d.

To obtain adult parasitoids for experimentation, mummies that formed within 24 h of one another were collected. These were placed in glass vials, closed with plastic lids, and modified to allow for the movement of air through a 1cm diameter hole covered in 75µm muslin. In each vial, a cotton wool wick saturated in a honey and water solution acted as a food and moisture source for emerging parasitoids. Parasitoids that emerged within 24 h of one another were used directly for experiments or placed at 10°C for 7 d in fresh vials as an acclimation treatment, again with a honey solution as a food and moisture source.

A preliminary investigation into the efficacy of *L. testaceipes* showed that 80% of aphids (n=50) exposed to parasitoids using the methods discussed above, were successfully parasitized and produced adult parasitoids. When the exposed aphids were acclimated at 10ºC for 7 d, it was possible to identify those that had been parasitized, due to a swollen appearance and a lighter colour (personal observations). Under this treatment, an increased proportion of aphids (n=50) selected for experimentation yielded adult parasitoids (90%). These two preliminary experiments served as a common control for expected levels of parasitism and adult emergence of non-acclimated and acclimated parasitoid life stages. Accordingly, for subsequent laboratory and field trials, 100% survival of parasitoids was recorded where there was 80% adult emergence from non-acclimated larval treatment groups and 90% emergence from acclimated larval treatment groups.

2.2.4 Rearing of Aphis fabae

Aphis fabae were reared under the same conditions as the parasitoids (23°C, 16 : 8LD) on *V. faba* plants. To obtain aphids of a particular age group, reproducing adults were placed on *V. faba* leaves within Blackman boxes **(**Blackman, 1971**)** and left for 24 h to reproduce. Following removal of the adults, the development of the nymphal aphids was monitored. Second to third-instar aphids were used for all experiments with *L. testaceipes* as research has shown that, whilst first instar aphids are smaller and thus easier to handle and less able to defend themselves from parasitoid attack (Gerling, 1990; Chau & Mackauer, 2001),

survivorship of the developing parasitoid is greater in second to third instar aphids (Liu & Stansly, 1996; Henry *et al*., 2005).

2.3 THERMAL BIOLOGY OF *NESIDIOCORIS TENUIS* AND *LYSIPHLEBUS TESTACEIPES*

2.3.1 Effect of temperature on rate of development

The effect of temperature on the developmental rate of *N. tenuis* and *L. testaceipes* was determined using a method similar to that described by Hart *et al*., (2002b) and Hatherly *et al*. (2008) in similar studies on non-native species with potential as biocontrol agents in Europe. Depending on the species, organisms were reared at a range of constant temperatures between 9.5 and 28°C. Individuals were monitored daily and time taken to complete each developmental stage from egg to adult was recorded. As the methods used for *N. tenuis* and *L. testaceipes* differed, full details of the techniques are given in Chapters 3 and 5.

The results were analysed using simple and weighted linear regression. The line was weighted using the inverse of the variance (Draper & Smith, 1981), overcoming any problems of variance heterogeneity and resulting in greater emphasis being placed on the values achieved at lower temperatures (Hart *et al*., 2002b; Olsen *et al*., 2003). The developmental threshold was estimated by identifying the point at which the weighted linear regression line crossed the x-axis.

2.3.2 Diapause in Nesidiocoris tenuis *and* Lysiphlebus testaceipes

It is evident that the capacity to arrest development in order to endure harsh conditions has contributed greatly to the success of the Class Insecta, enabling them to exploit a wide variety of habitats from the tropics to the poles (Denlinger, 2002). Thus the diapausing ability of *N. tenuis* and *L. testaceipes* will be investigated.

The methods used to assess diapausing ability varied considerably between the two subject species and thus full details are given in chapters 3 and 5.

2.3.3 Supercooling points of Nesidiocoris tenuis *and* Lysiphlebus testaceipes

The SCP of each individual was identified using a technique described by Bale *et al*. (1984). Organisms were attached to type K exposed wire thermocouples using a small amount of Vaseline grease. These were then placed individually into size 3 Beem capsules (Agar Scientific Ltd, UK) and each capsule was placed into a boiling tube suspended in a programmable alcohol bath (five capsules per boiling tube). The temperature was reduced at 0.5°C min^{-1} from the rearing or acclimation temperature to -30ºC. The SCP of each sample was detected by the exotherm that occurs when an insect freezes. Experiments were carried out on four to six treatment groups (see chapters 3 and 5 for details), with sample sizes varying from 39 to 53.

2.3.4 Lower lethal temperature in Nesidiocoris tenuis *and* Lysiphlebus testaceipes

Fifty individuals from each treatment group were placed individually into size 3 Beem capsules. Ten capsules were placed in each of 5 boiling tubes suspended in a programmable alcohol bath (Haake Phoenix 11 P2, Thermo Electron Corp., Karlsruhe, Germany). The temperature was reduced at 0.5°C min^{-1} from the rearing (23ºC) or acclimation temperature (10°C) to a range of temperatures between -10 and -15ºC for mirids and -5 and -30°C for parasitoids, with 50 individuals exposed at each temperature. These temperatures were selected on the basis of preliminary experiments known to encompass a range of mortalities up to 100%. Insects were held at the minimum temperature for 15 min, after which the temperature was raised up to the rearing or acclimation temperature, again at a rate of 0.5°C min^{-1}.

Control samples of 50 of each life stage were placed individually into size 3 Beem capsules. These were also held in boiling tubes suspended in the alcohol bath (10 capsules per tube) and the temperature was reduced from the rearing temperature (23°C) to 15ºC at 0.5°C min^{-1}. The minimum temperature of 15°C was maintained for 2.25h for mirids and 4 h for parasitoids before increasing again to the rearing temperature at 0.5ºC min^{-1}. The period of

time for which the species were held at 15°C ensured that total time spent in the alcohol bath was equivalent to or greater than that of the treated groups.

Details of mortality assessment for the treated and control groups in *N. tenuis* and *L. testaceipes* are given in chapters 3 and 5.

The lower lethal temperature experiments were conducted on the same treatment groups as the SCP experiments. Similar methods have been used to estimate the lower lethal temperatures in previous studies on non-native biocontrol agents (Hart *et al.*, 2002a,b; Hatherly *et al.*, 2003, 2008; Hughes *et al.*, 2009).

2.3.5 Lower lethal time in Nesidiocoris tenuis *and* Lysiphlebus testaceipes

Samples were set up as described in chapters 3 and 5. For each treatment group, following exposure to 10°C for 1 h to overcome the possibility of mortality due to cold shock, samples were placed at -5, 0 and 5ºC. At set time intervals, identified by preliminary experiments, samples of each treatment group were removed from each exposure temperature and held for 1 h at 10°C to prevent mortality due to heat shock (a sample consisted of four replicates of 10 individuals for mirids and five replicates of 10 individuals for parasitoids). The mortality of the organisms was assessed, after 72 h, in the same way as for the lethal temperature experiments.

A control sample of 50 of each life stage (adults and nymphs for mirids and parasitized second instar aphids, mummies, and adults for parasitoids) was set up as described for the treatment groups, with the addition of *E. kuehniella* eggs as a food source for mirids. The method used to assess the percentage mortality in mirid and parasitoid control groups varied considerably and thus full details are given in chapters 3 and 5.

Methods used to estimate the lower lethal times were again based on previous studies (Hart *et al.*, 2002a,b; Hatherly *et al.*, 2003, 2008; Hughes *et al.*, 2009) and the experiments were carried out on the same treatment groups as for SCP and lethal temperature experiments.

2.3.6 Nesidiocoris tenuis *and* Lysiphlebus testaceipes *field trial*

All field trials were carried out in a sheltered location in the University of Birmingham grounds. However, as experimental details of the individual field trials varied considerably, methods are described in full in chapters 3 and 5.

2.3.7 Host range experiments

Host range experiments were only carried out for *L. testaceipes* and thus details of the methods are described in chapter 5.

2.3 8 Statistical analysis

The results of both the lower lethal temperature and lower lethal time experiments were analysed using Probit analysis (Finney, 1971), and the temperatures resulting in 10, 50 and 90% mortality were calculated ($LTemp_{10,\ 50,\ 90}$). Significant differences in mortality were identified by non-overlapping fiducial limits (Hart *et al.*, 2002a,b; Hatherly *et al.*, 2003, 2008; Hughes *et al.*, 2009).

The field mortality data were analysed by a binary logistic regression model and the alpha level was set at 0.05.

Statistical analysis of the developmental data and SCP results varied considerably and thus full details of individual species are described in chapters 3 and 5.

2.4 THERMAL ACTIVITY THRESHOLDS OF *NESIDIOCORIS TENUIS* AND *LYSIPHLEBUS TESTACEIPES*

2.4.1 Experimental system

The equipment comprised a hollow aluminium block with a circular ‘arena’ in which the organisms were placed (Fig. 2.1). To prevent the mirids and mites from climbing up the

sides of the arena, the walls were coated in Fluon (Blades Biological, Edenbridge, UK) and the arena was covered in a thin sheet of Perspex. The arena was temperature controlled via connection to an alcohol bath from which heated or cooled fluid was circulated through channels within the block. The behaviour of the organisms was recorded using a digital video camera (Infinity 1-1, Lumenera Scientific, Ottawa, Canada) with a macro lens (Computar MLH-10X, CBC Corp., U.S.A.), providing a permanent record of the experiments for retrospective analysis. The temperature within the arena was monitored continuously using a thermocouple inserted into the sidewall of the arena which was attached to an electronic thermometer linked to the video recording software (Studio Capture DT, Studio86Designs, Lutterworth UK) so that the temperature was displayed in the field of view of the recorded images during 'playback'. Arenas of three different sizes were used for the experiments: 40 mm diameter by 7.5 mm depth for adult mirids and parasitoids, 25 mm by 7.5 mm for nymphal mirids and aphids, and 15 mm by 7.5 mm for the mites.

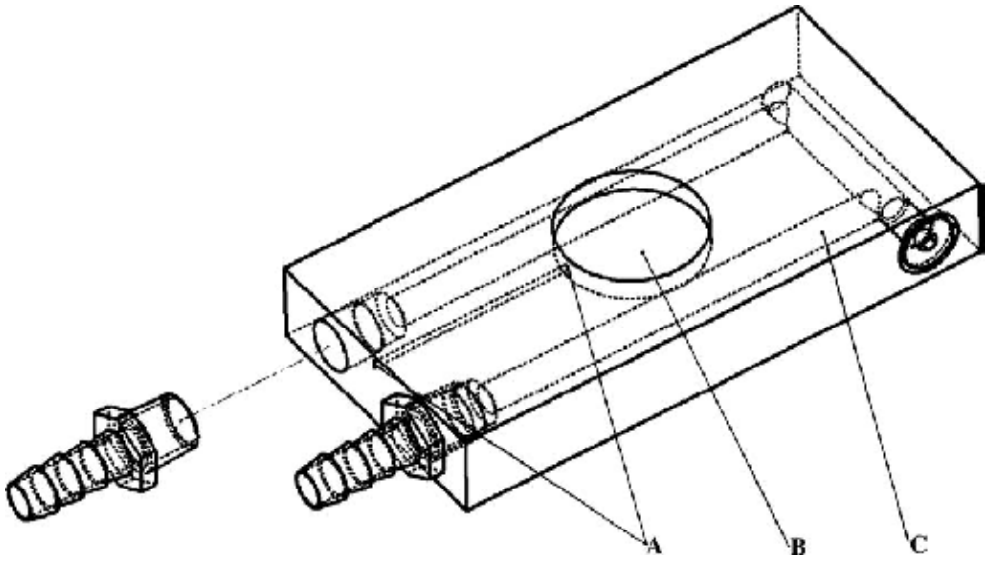


Figure 2.1: Design of the aluminium block system showing: A: passage for the thermocouple; B: arena; C: channel bored into the block to allow heated or cooled fluid to be pumped around the block (from: Hazell *et al.*, 2008).

2.4.2 Thermal thresholds

When an insect or mite is cooled, one of the first observable physiological responses is a decrease in walking speed. When cooling is continued, a temperature is reached at which the organism loses locomotory function; this is the insect's critical thermal minimum (CT_{min}) (Cowles & Bogert, 1944). This temperature has been variously defined as the limit for escape from a hostile environment (Fry, 1967), the temperature at which an insect loses muscle function (Klok & Chown, 2003; Terblanche *et al.*, 2005), is unable to right itself (Renault *et al.*, 1999; Gilbert *et al.*, 2001; Castaneda *et al.*, 2004,2005), or is unable maintain coordinated movement (Sinclair *et al.*, 2006). A further reduction in temperature renders the organism completely immobile (i.e. the temperature at which there is a 'last twitch' of an antenna or leg, a state known as chill coma). In chapter 4, the temperatures at which these two behaviours are observed are coded T1 and T2, respectively. It should be noted, however, that the terms CT_{min}, chill coma and cold torpor have been used more or less synonymously in various papers. The distinction that can be made between the CT_{min} and chill coma in this project is related mainly to the fact that the insects are video-recorded and hence the 'true' chill coma (last movement of an appendage) temperature can be accurately determined, whereas with all other methods, this would not be possible.

Chill coma is a reversible condition from which an insect can usually recover when warmed. Following chill coma, the first response to an increase in temperature is the movement of an appendage, a stage referred to as chill coma recovery. A further increase in temperature will result in the restoration of locomotor function, known as 'activity recovery'. In chapter 4 the temperatures at which these behaviours are observed are coded T3 and T4, respectively.

Similar responses are seen when an organism is heated. Following an initial increase in walking speed, a temperature is reached at which the organism loses the ability to move in a coordinated manner. This state, originally described by Cowels and Boggert (1944), is the critical thermal maximum (CT_{max}) (see Lutterschmidt & Hutchison, 1997 for a review). At a higher temperature, the organism then enters heat coma. The temperatures at which these behaviours are observed are coded T5 and T6, respectively. Often, the heat coma temperature and the upper lethal limit do not differ significantly (Hazell *et al.*, 2009), suggesting that heat coma, unlike chill coma, is an irreversible state leading to mortality and

further, means that it is usually impossible to determine either a heat coma recovery or upper activity recovery temperature.

2.4.3 CTmin and chill coma

Temperature was reduced from the rearing or acclimation temperature (23° and 10°C, respectively) to -2° for mirids and mites, -5° for aphids and -15°C for parasitoids, at a rate of 0.2°C min^{-1}. These minimum temperatures were selected on the basis of preliminary experiments known to result in 100% entry into chill coma in all species. The video recording was analysed and the temperatures at which each individual reached CT_{min} (T1) and entered chill coma (T2) were recorded. Thirty individuals were monitored for the mirid and mite treatment groups and between 30 and 40 individuals for the parasitoid and aphid treatment groups.

2.4.4 Chill coma recovery and activity recovery

Chill coma recovery and activity recovery were observed in a fresh sample of organisms. Temperature was reduced from the rearing or acclimation temperature (23°C and 10°C, respectively) at a rate of 0.5°C min^{-1} to -2° for mirids and mites, -5° for aphids and -15°C for parasitoids, temperatures which ensured 100% entry into chill coma in the respective species. Organisms were held at this temperature for 5 min before being warmed to the rearing or acclimation temperatures at a rate of 0.2°C min^{-1}. The video recording was analysed and the temperatures at which each individual showed chill coma recovery (T3) and activity recovery (T4) were recorded. As for the chill coma experiments, 30 individuals were monitored for the mirid and mite treatments and between 30 and 40 individuals for parasitoid and aphid treatments.

2.4.5 CT_{max} and heat coma

Temperature was increased at a rate of 0.2°C min^{-1} from the rearing or acclimation temperature (23°C and 10°C, respectively) to 55°C. This maximum temperature was selected on the basis of preliminary experiments known to induce heat coma in all individuals. The

video recording was subsequently analyzed and the temperatures at which the subjects entered CT_{max} (T5) and heat coma (T6) were recorded. Again, 30 individuals were monitored for the *N. tenuis* and *T. urticae* treatment groups and 30 to 36 individuals were monitored for the *L. testaceipes* and *A. fabae* treatment groups.

2.4.6 Walking speed

The temperature of the arena was reduced from the rearing or acclimation temperature (23°C and 10°C, respectively), to 0°C for mirids and mites and -2.5°C for parasitoids and aphids, at 2.5°C intervals. At each interval, the temperature was held for 5 min and movement of the organisms around the arena was recorded. The video recording was analysed using StudioMeasure. At each temperature, the time taken for an individual to move a measured distance across the arena in a straight line (20-35 mm for adult mirids and parasitoids, 10-20 mm for nymphs and aphids and 5-10 mm for mites) was recorded and, from these data, the average walking speed at each temperature was calculated. Thirty individuals were monitored for each age and treatment group.

2.4.7 Statistical analysis

For each treatment in the thermal threshold experiments, the distribution that best described the data was determined using the distribution fitting procedure (Distribution ID) in MINITAB 15 (Minitab Inc. 2007). Using the appropriate distribution, parametric distribution analysis was performed in Minitab 15, allowing differences in both the scale and location of the data to be identified (see Hazell *et al.,* 2008 for details). Individual comparisons were made using the Bonferroni 95% Confidence intervals.

The walking speed data were analysed using the Scheirer-Ray-Hare extension of the Kruskal-Wallis test (a non-parametric two-way ANOVA design). The alpha level was set at 0.05. For the *post-hoc* Mann-Whitney tests, the alpha levels at which differences were considered significant were Bonferroni adjusted for multiple comparisons.

CHAPTER 3

Thermal biology and establishment potential in temperate climates of the predatory mirid *Nesidiocoris tenuis*.

3.1 ABSTRACT

Nesidiocoris tenuis Reuter (Hemiptera: Miridae) is a polyphagous mirid currently used for the control of leafminers, thrips, whitefly and spider mites in Mediterranean regions to which it is indigenous. This study investigates the establishment potential of *N. tenuis* in cool temperate climates typical of northern Europe through assessment of its thermal biology and low temperature tolerance in laboratory and field experiments. The developmental threshold of *N. tenuis* was estimated to be 12.9°C with no indication of ability to diapause. Supercooling points of the non-acclimated and acclimated adults and nymphs of the mirid were between -17.6° and -21.5°C and the $LTemp_{50}$ was approximately -12°C, indicating a high level of pre-freeze mortality. The $LTime_{50}$ at 5°C was 9 d and 100% mortality occurred after less than 4 weeks of winter field exposure. Collectively these data suggest that *N. tenuis* is unlikely to establish in northern Europe and would therefore have little or no non-target effects on native species in such regions.

3.2 INTRODUCTION

Biological control has long been viewed as a 'green' approach to pest management (Cory & Myers, 2000) and, by implication, having less risk of negative environmental impacts than pesticides. Yet, as van Lenteren *et al.* (2006) have noted, despite the large number of introductions worldwide in the last 120 years, with very few reports of impacts on non-target organisms, there has been an increasing trend towards the regulation of non-native species. Accompanying this development, there has been an identified need to produce and evaluate research methodologies to underpin an effective system of environmental risk assessment

(ERA) that does not compromise the health of the environment, but at the same time does not impose unnecessary or unaffordable costs on companies that produce beneficial organisms.

The situation in Europe is complicated by the fact that there is no EU-wide policy of regulation or risk assessment. Across neighbouring member states there are some countries with regulation (e.g. UK, Denmark, Hungary and Sweden) and some without (e.g. France, Greece and Italy) (Loomans, 2007); but, of course, there is nothing to prevent an organism released in a country without regulation moving into a country where it would not have been released, as has occurred with the predatory ladybird *Harmonia axyridis* Pallas (Coleoptera: Coccinellidae) (Majerus *et al.*, 2006). Additionally, in those countries with some system of regulation, there is often no consistency in the information required when seeking a licence to release a non-native species. Also there is no evidence that some of the early regulatory systems may have been inadequate to identify species with undesirable establishment potential in the countries of release. Additionally, it was thought that species originating in tropical and Mediterranean regions would lack the necessary adaptations required for survival in temperate or colder climates. Under this supposition, 'climate origin' was used as a proxy for direct assessment of cold tolerance. However, such ideas have been called into question following the discovery of populations of the glasshouse biological control agent *Neoseiulus californicus* McGregor (Acari: Phytoseiidae) outside glasshouse environments in the UK (Jolly, 2000) and frequent sightings in winter of the predatory mirid *Macrolophus caliginosus* Wagner (Hemiptera: Miridae) (Hart *et al.*, 2002b). However, there is currently no documentation of any negative impacts of these species on native fauna.

A system of risk assessment of biological control agents has been described by van Lenteren and Loomans (2006), in which a stepwise analysis is applied to candidate species, usually in the order of establishment, host range and dispersal, though there is flexibility in the sequence of testing. For example, with releases into glasshouses in northern Europe, if escaping agents can be shown to be incapable of outdoor establishment because of climatic constraints, it would not normally be necessary to investigate other components such as host range or dispersal. Conversely, if an agent was intended for outdoor biocontrol in Mediterranean areas, it is likely that year-round development and reproduction would occur. Hence, the risk assessment would focus mainly on host range. Against this background, this chapter investigates the cold tolerance of *N. tenuis* as part of an assessment of its establishment potential in cool temperate climates that occur throughout northern Europe. In a wider

context, the data will be used to examine the relationship between the laboratory survival at 5°C and winter field survival as part of the ERA of non-native biocontrol agents (Hatherly *et al.*, 2005, 2008).

3.3 MATERIALS AND METHODS

3.3.1 Rearing of Nesidiocoris tenuis

The rearing methods of *N. tenuis* are described in chapter 2, section 2.2.1.

3.3.2 Effect of temperature on rate of development

Fifty newly moulted adult mirids were placed on each of seven tobacco plants in separate ventilated containers at 23°C, 16:8 L:D. The mirids were left on the plants for 24 h to mate and lay eggs. Following removal of the mirids from tobacco, the individual plants were placed in incubators at 15.5°, 20°, 25°, 27.5°, 30° and 32°C, 16:8 LD. The plants were monitored daily for emerging nymphs. As the nymphs emerged, they were placed individually into glass vials on a 1cm layer of agar (2%) (Oxoid Ltd, UK) covered with a circular disk of tobacco leaf sprinkled with *E. kuehniella* eggs to provide both a food and moisture source and maintained at the same temperatures. The vials were closed with plastic lids, modified to allow for the movement of air through a 1cm diameter hole covered in 75 µm muslin. The vials were changed every 3 d to ensure that a fresh supply of *E. kuehniella* eggs was available to the mirids at all times, and to prevent the growth of fungal spores that could entangle the insects and affect development times. The vials were checked daily and the time taken to complete each developmental stage from egg to adult was recorded. The sample size at each temperature varied from 32 to 72 reflecting the different number of eggs laid on each plant, and the number of emerging nymphs.

The results were analysed using the method described in section 2.3.1

3.3.3 Diapause

Fifty newly moulted adults were placed on each of nine tobacco plants within secure, ventilated plastic containers at 23°C, 16:8 LD. The mirids were left on the plants for 48 h to mate and lay eggs. Following removal of the mirids from the tobacco plants, six plants were transferred to short day conditions (20°C, 12:12 LD), and three remained at 23°C, 16:8 LD. The plants were held under these conditions until the emerging nymphs had moulted to adult. The resulting adults were placed on fresh tobacco plants, two males and one female per plant. Fifteen plants with *N. tenuis* from 23°C, 16:8 LD and 15 plants with *N. tenuis* from 20°C, 12:12 LD were retained at the same rearing conditions and a further 15 plants with insects from the short day conditions were transferred to 20°C, 16:8 LD.

The pairs were left to mate and lay eggs on tobacco and transferred onto fresh plants at weekly intervals for 28 d. The tobacco plants were monitored daily for nymphal emergence. Any emerging nymphs were recorded and removed from the plants. The counts continued on each plant until there were 5 d free from emerging nymphs.

3.3.4 Supercooling points

Experiments were carried out as described in section 2.3.3 on four treatment groups: adults and second to third instar nymphs in non-acclimated and acclimated states. The sample size for each treatment group varied from 48 to 53.

3.3.5 Lower lethal temperature

The SCP experiments were carried out as described in the chapter 2, section 2.3.4, on the same four treatment groups as the SCP experiments.

Mortality in the treated and control groups was recorded 72 h after exposure. The mirids were included in the mortality count if they were unable to walk in a coordinated manner or to right themselves.

3.3.6 Lower Lethal Times

For each adult and nymphal treatment (non-acclimated and acclimated), five mirids were placed in a glass vial on a 1 cm layer of agar (2%), covered with a circular piece of filter paper to provide a moisture source. The nymphs were placed in 10 ml vials and the adults in 20 ml vials. A total of 120 vials were set up for each treatment group. The lethal time experiment was then carried out as described in the chapter 2, section 2.3.5.

A control sample of 50 adults and 50 nymphs was maintained for the duration of the experiment at 15°C within glass vials as described for the treatment groups but with the addition of *E. kuehniella* eggs as a food source. The mortality of the control was assessed 72 h after the completion of the experiment.

3.3.7 Adult and nymph field mortality

The field experiment was conducted from 11th of November to 4th December 2007. For all adult and nymphal treatments, five mirids were placed in a glass vial on a 1 cm layer of agar (2%), covered with a circular piece of filter paper to provide a moisture source. A total of 140 vials were set up for each treatment group (non-acclimated and acclimated, fed and un-fed adult and nymph *N. tenuis*), allowing for 5 replicates of 10 mirids to be sampled over 14 time intervals. The fed groups were provided with *E. kuehniella* eggs sprinkled onto the filter paper. The vials were closed with plastic lids, modified to allow for the movement of air through a 1 cm diameter hole covered in 75 µm muslin. The vials were then placed in secure, ventilated plastic boxes in the field in a sheltered location at the University of Birmingham, UK. A Tinytalk ® datalogger (Gemini, UK) placed within each box recorded temperature throughout the experiment. Plastic trays (45 × 35 cm) were placed on top of each box to provide protection from direct sunlight.

A control sample of 50 adults and nymphs was maintained for the duration of the field trial at 15°C, with a food and moisture source provided, and mortality assessed at 3 d intervals. This temperature was selected following preliminary investigations indicating that at 10°C, mirids had difficulty in completing the moulting process.

All treatments were transferred into new vials with fresh agar at 3 to 4 d intervals with the fed mirids receiving a fresh supply of *E. kuehniella* eggs at the same time. This precaution was taken to prevent the formation of mould spores on the agar that could entangle the mirids and result in increased mortality. The vials containing the un-fed mirids were checked at 2 d intervals to remove any dead insects which may also have served as a food source for the survivors.

For each treatment, samples were taken from the field at regular intervals and mortality recorded after 72 h using the same criteria as for the lethal temperature and lethal time experiments.

3.3.8 Statistics

The developmental times from egg to adult in male and female mirids were analysed using a non-orthogonal ANOVA with normally distributed error and the residuals were tested for normality. Differences in rate of development of the sexes were considered significant when $P < 0.05$.

The SCP results were also analysed using non-orthogonal ANOVA with normally distributed error and again the residuals were tested for normality. Multiple comparisons were made using the Tukey method where the alpha level was set at 0.05.

The lower lethal time, lower lethal temperature and field mortality data were analysed using methods described in the chapter 2, section 2.3.8.

3.4 RESULTS

3.4.1 Developmental threshold

The mean number of days (±1 SE) for development of the egg and five nymphal instars are shown in Table 3.1. Developmental time for each life stage decreased with increasing

temperature. No differences in the developmental times of male and female *N. tenuis* were identified ($F_{1, 329} = 0.11$; $P = 0.74$).

Table 3.1: Effect of rearing temperature on the developmental time (mean days ± SE) of male (M) and female (F) *Nesidiocoris tenuis* (16:8 LD).

Temp. (°C)	N	% survival to adult	Egg	1st instar	2nd instar	3rd instar	4th instar	5th instar	Egg to adult (%)
15.5	44	52.3	28.3 ± 0.2	12.7 ± 0.2	9.7 ± 0.2	10.6 ± 0.1	10.9 ± 0.2	21.1 ± 0.5	M 94.2 ± 0.3 (56.5) F 91.7 ± 1.0 (43.5)
18.0	68	73.5	18.9 ± 0.1	6.8 ± 0.1	6.2 ± 0.1	5.2 ± 0.1	5.9 ± 0.1	11.2 ± 0.2	M 53.6 ± 0.6 (46.0) F 53.8 ± 0.5 (54.0)
20.0	72	97.2	16.9 ± 0.1	5.6 ± 0.1	3.8 ± 0.1	3.9 ± 0.1	4.4 ± 0.1	6.9 ± 0.1	M 41.4 ± 0.4 (50.0) F 41.6 ± 0.2 (50.0)
25.0	52	94.2	9.4 ± 0.1	2.9 ± 0.1	2.2 ± 0.1	2.1 ± 0.0	2.7 ± 0.1	3.9 ± 0.1	M 23.0 ± 0.2 (49.0) F 23.0 ± 0.2 (51.0)
27.5	49	98.0	7.3 ± 0.1	2.5 ± 0.1	1.3 ± 0.1	1.4 ± 0.1	2.1 ± 0.1	3.1 ± 0.0	M 17.8 ± 0.2 (47.9) F 17.5 ± 0.2 (52.1)
30.0	65	100	6.3 ± 0.1	2.4 ± 0.1	1.5 ± 0.1	1.2 ± 0.1	1.4± 0.1	2.6 ± 0.1	M 15.3 ± 0.1 (49.2) F 15.3 ± 0.1 (50.8)
32.0	32	100	5.9 ± 0.1	2.2 ± 0.1	1.2 ± 0.1	1.1 ± 0.1	1.0 ± 0.1	2.1 ± 0.1	M 14.0 ± 0.2 (56.3) F 14.1 ± 0.1 (43.7)

The developmental threshold derived from the weighted linear regression was estimated to be 12.9°C (Fig. 3.1) with a day degree requirement for development from egg to adult of 275 (the weighted linear regression gave a better fit to the values achieved at lower temperatures due to the greater variance in the data at higher temperatures). At 20ºC, newly emerged adults were observed to mate and lay eggs within 24 – 48 h.

To determine the likely annual voltinism of *N. tenuis* in the UK over the past 15 years, the available thermal budget was calculated by subtracting the developmental threshold from the mean daily temperature for each day, and then summing these values to obtain an annual total. With knowledge of the day degree requirement per generation, these data were then used to estimate the annual voltinism under outdoor conditions (Table 3.2). From these data it is evident that over the 15 year period of 1993 to 2007, *N. tenuis* would never have more than two full generations per year outdoors in the UK.

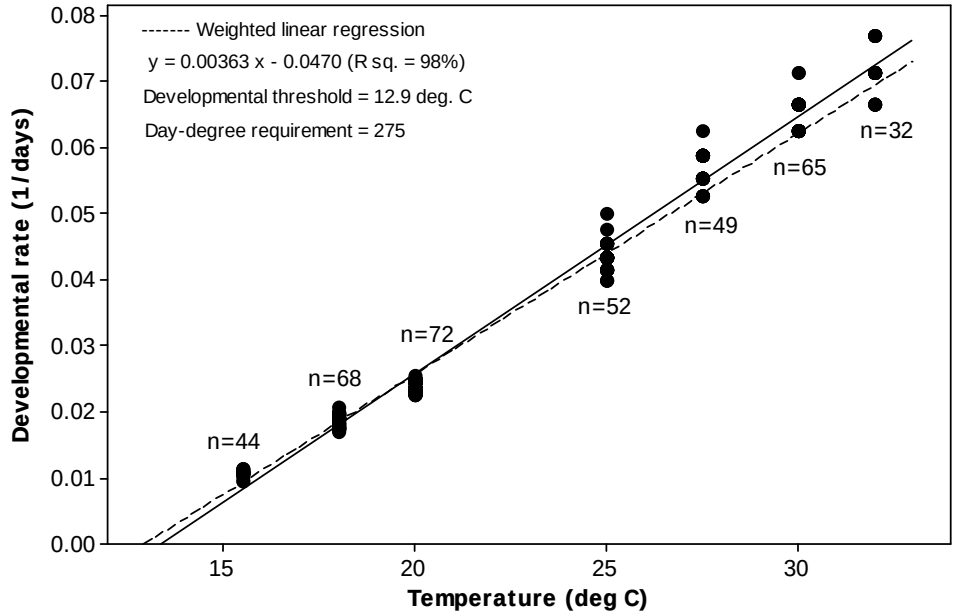


Figure 3.1: Developmental rate of *Nesidiocoris tenuis* from egg to adult at seven constant temperatures. Lines fitted by simple linear regression (solid line) and weighted linear regression (broken line). Developmental threshold determined by extrapolation of the fitted line (weighted linear regression) to the x-axis.

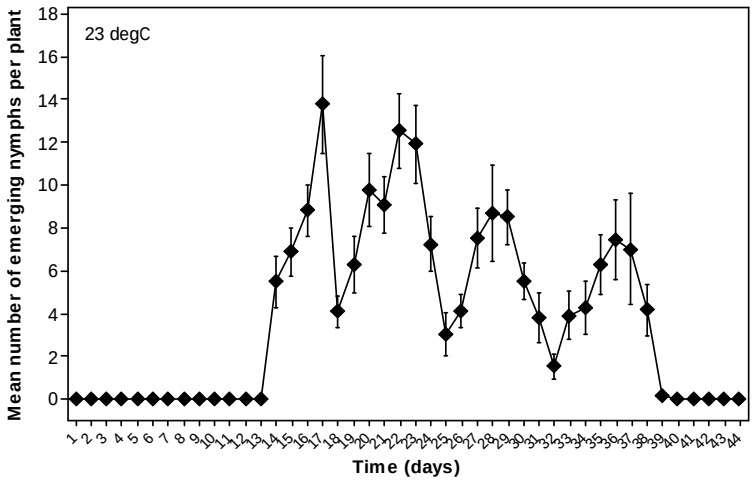
Table 3.2: Estimated annual voltinism of *Nesidiocoris tenuis* over 15 years in Birmingham, UK.

Year	Annual available day-degrees	Theoretical number of generations per year
1993	246	0.9
1994	330	1.2
1995	511	1.8
1996	324	1.2
1997	413	1.5
1998	302	1.1
1999	366	1.3
2000	330	1.2
2001	381	1.4
2002	322	1.2
2003	468	1.7
2004	416	1.5
2005	403	1.4
2006	533	1.9
2007	288	1.0
Mean	**376**	**1.4**

3.4.2 Diapause

Under long day control conditions (23°C, 16:8 LD), emergence of nymphs was continuous from day 12 to day 39. Movement to a fresh plant appeared to increase egg-laying, as evidenced by the patterns of nymphal emergence in Figure 3.2a, with peaks of emergence occurring at approximately 7 d intervals. These peaks of emergence appeared to coincide with the transfer of adult pairs to new tobacco plants, possibly due to the reduction in oviposition sites on the previous plants.

(a)



(b)

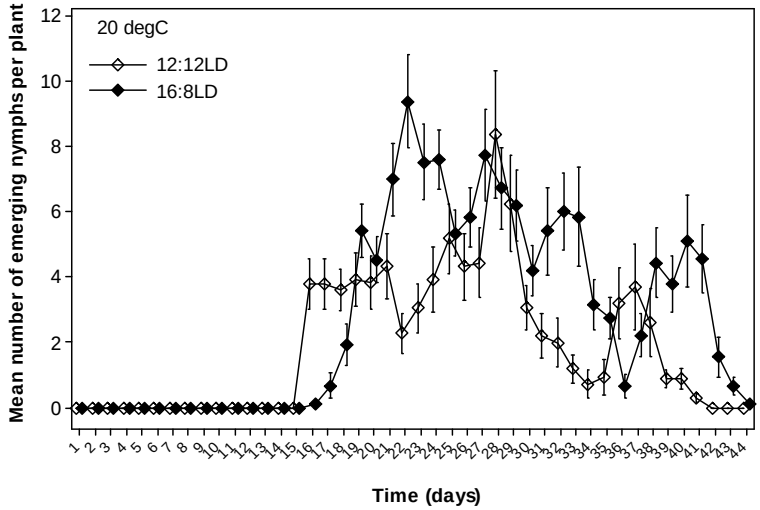


Figure 3.2: (a) Mean number of emerging *Nesidiocoris tenuis* nymphs per plant per day (± SE) at 23°C, 16:8 LD as a result of egg laying by female adult mirids reared from eggs under the same conditions; (b) Mean number of emerging *Nesidiocoris tenuis* nymphs per plant per day (± SE) at 16:8 LD and 12:12 LD at 20°C as a result of egg laying by female adult mirids reared from eggs under short day conditions (20°C, 12:12 LD).

The pattern of nymphal emergence (and therefore previous egg laying) of *N. tenuis* reared under short day conditions (20°C, 12:12 LD) (Fig. 3.2b) was similar to that of control conditions (Fig. 3.2a). The 2 d difference in the start of nymphal emergence between the control and the two treatment groups can be attributed to the 3°C difference in rearing conditions for these two populations. One hundred percent of females (n=19) reared under short day conditions laid eggs on transferral to a fresh plant and long day conditions (20°C, 16:8 LD) and 90% of the females (n=20) retained under the short day conditions also laid eggs on transfer to a fresh plant (Fig. 3.2b).

3.4.3 Supercooling points

The mean SCP of the four experimental groups differed by only 3.9°C, but some of these differences were significant (Table 3.3). Nymphal mirids had a significantly lower SCP than the adults ($F_{1,197}$=118.15; P<0.001) and the acclimation regime served to increase the SCP of the groups significantly ($F_{1,197}$=11.56; P<0.001), indicating an inability to acclimatise to a lower temperature. There was no interaction between the age group of the mirids and the acclimation regime ($F_{1,197}$=2.18; P = 0.14).

Table 3.3: Mean (±1 SE) and range of SCPs of non-acclimated and acclimated nymphal and adult *Nesidiocoris tenuis.*

Experimental group	N	Mean ± SE (°C)	Range (°C)
Non-acclimated nymphs	50	-21.5 ± 0.4	-22.2 to -20.7
Acclimated nymphs	50	-20.9 ± 0.3	-21.6 to -20.3
Non-acclimated adults	53	-18.9 ± 0.2	-19.3 to -18.6
Acclimated adults	48	-17.6 ± 0.1	-17.8 to -17.4

3.4.4 Lower lethal temperature

The lethal temperatures for 10, 50 and 90% mortality ($LTemp_{10, 50, 90}$) for the four treatment groups are shown in Figure 3.3. No mortality was recorded in the control. There was no

difference in mortality between the non-acclimated and acclimated nymphs ($LTemp_{50}$ -13.2° and -13.1°C, respectively), nor between the non-acclimated and acclimated adults ($LTemp_{50}$ -12.1° and -12.2°C, respectively), as indicated by overlapping fiducial limits. However, the $LTemp_{50}$ and $LTemp_{90}$ were significantly lower for nymphs than adults. For all treatment groups, the $LTemp_{50}$ was markedly higher than the mean SCP.

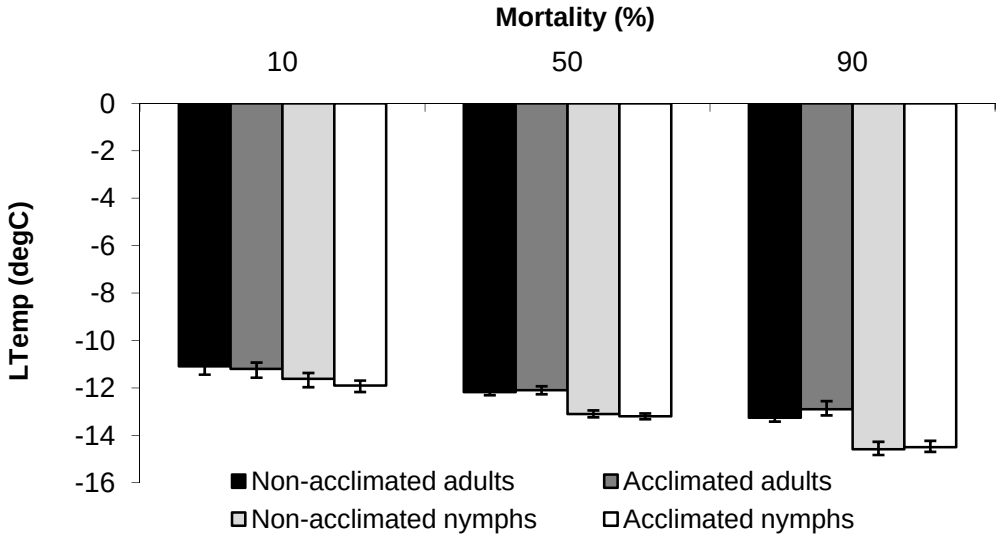


Figure 3.3: $LTemp_{10, 50}$ and $_{90}$ (± 95% fiducial limits) of non-acclimated and acclimated adult and nymphal *Nesidiocoris tenuis.*

3.4.5 Lower lethal times

The lethal times for non-acclimated and acclimated adult and nymph *N. tenuis* at 5º, 0° and -5°C for 10, 50 and 90% mortality ($LTime_{10, 50, 90}$) are shown in Figure 3.4. In the control there was 4% mortality of nymphs and 2% mortality of adults. When adults and nymphs were acclimated for 7 d at 10°C there was no increase in survival when exposed at 5°C (as indicated by overlapping fiducial limits) and a decrease in survival at 0°C compared with the non-acclimated population (as indicated by non-overlapping fiducial limits). There was, however, an increase in cold tolerance in acclimated adults at -5°C. In general, there was no difference in cold tolerance between adults and nymphs. The only exception was observed at 5°C, where adults were more cold hardy than nymphs for 10, 50 and 90% mortality. The $LTime_{50}$ at 5°C for non-acclimated and acclimated adults and non-acclimated and acclimated nymphs were 8.9, 8.3, 6.1 and 5.5 d, respectively.

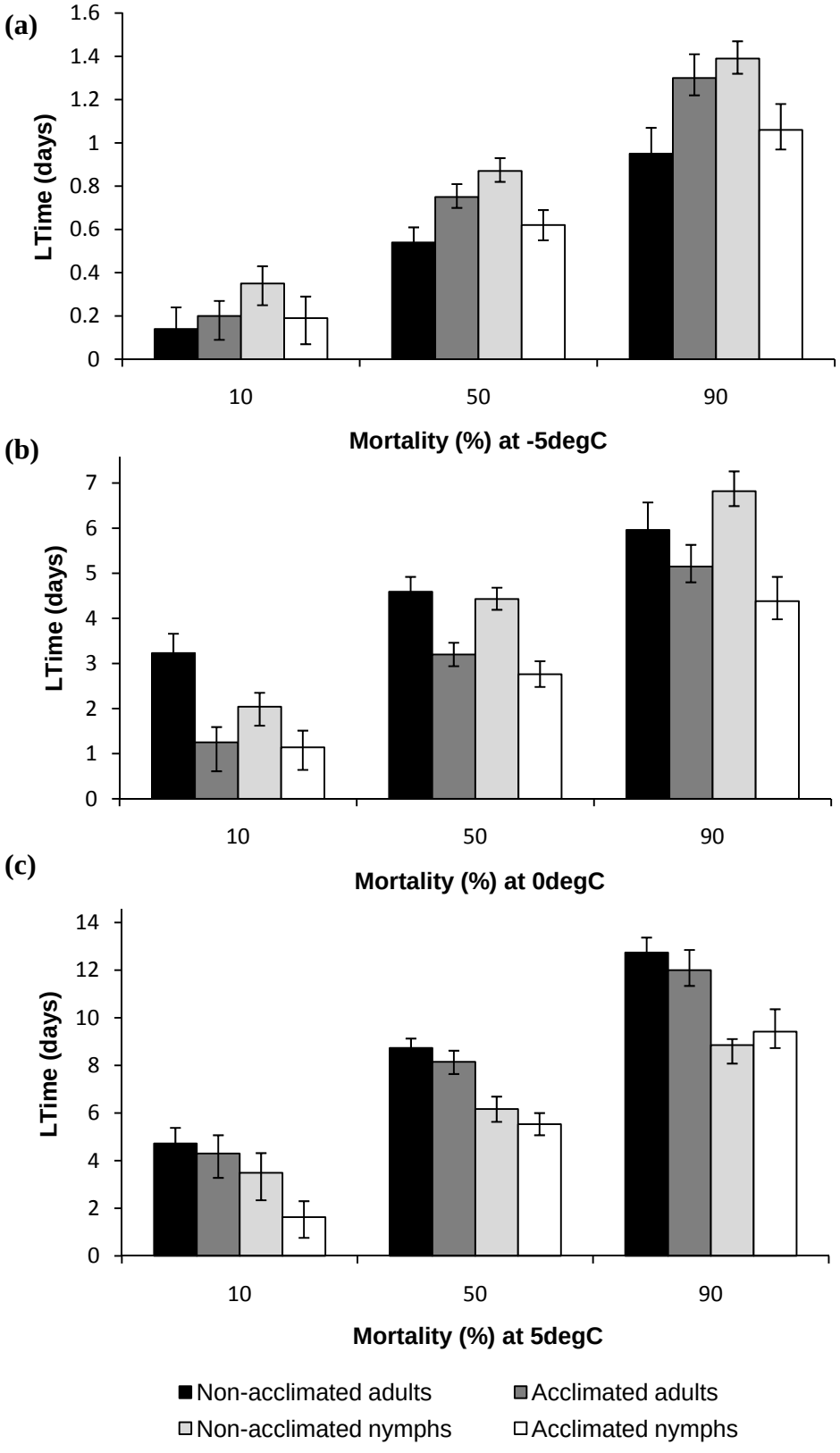


Figure 3.4: LTime $_{10,50}$ and $_{90}$ (± 95% fiducial limits) at (a) -5°, (b) 0° and (c) 5°C for non-acclimated and acclimated adult and nymphal *Nesidiocoris tenuis.*

3.4.6 Field mortality

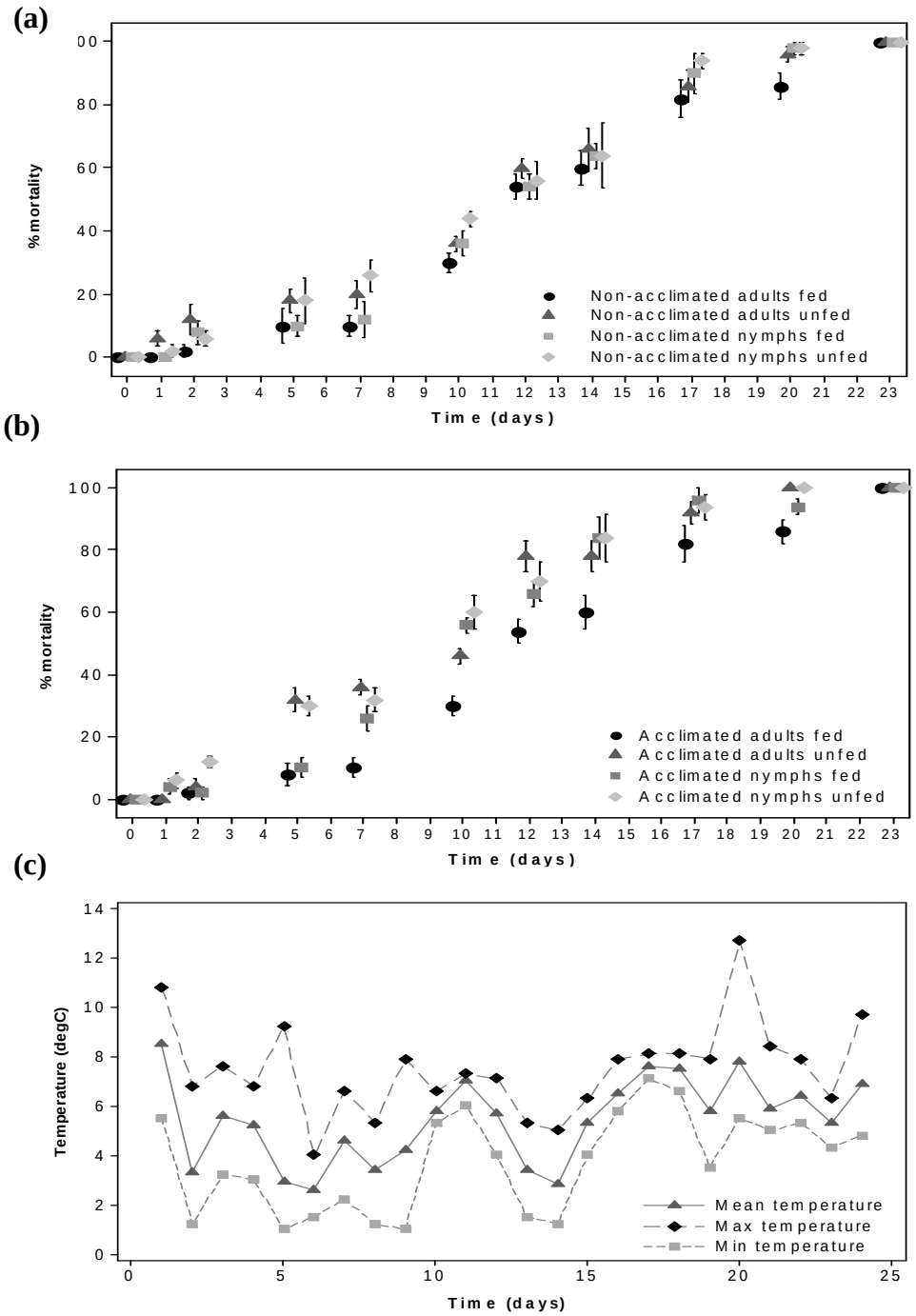


Figure 3.5: Mortality (mean ± SE) of (a) non-acclimated and (b) acclimated adult and nymphal *Nesidiocoris tenuis* with and without food over the same period. (c) Minimum, maximum and mean temperatures experienced by adult and nymphal *Nesidiocoris tenuis* in the field from 11th November to 4th December 2007.

The mortality rates of non-acclimated and acclimated fed and unfed adults and nymphs of *N. tenuis* in the field from the 11th November to 4th December 2007 are shown in Figure 3.5a and 3.5b, respectively. The corresponding field temperatures are shown in Figure 3.5c. The averaged daily mean, maximum and minimum temperatures during the field trial were 5.4º, 12.7º and 1°C, respectively. Although the mirids were never exposed to sub-zero temperatures, 100% mortality was recorded for the acclimated unfed treatments of adults and nymphs after 20 d and after 23 d for all of the remaining treatment groups. By comparison, there was 5% mortality of the nymphs and 10% mortality of the adults in the controls. Adult mirids had a significantly lower mortality than nymphal mirids in the field ($Z=-2.64$; $DF=1$; $P=0.008$) and the provisioning of *E. kuehniella* eggs also served to increase survival ($Z=4.33$; $DF=1$; $P<0.001$); however, exposure of the mirids to 10°C for a week prior to field exposure resulted in an increase in mortality ($Z=3.18$; $DF=1$; $P<0.001$), again confirming the absence of any acclimatory ability in this insect.

3.5 DISCUSSION

The establishment potential of a species can be affected by a number of factors, although in temperate climates, temperature can have a major influence on key processes such as development and survival. This chapter focuses on indices of cold tolerance of *N. tenuis* in laboratory and field experiments.

The developmental threshold of *N. tenuis* was estimated to be 12.9°C with a day degree requirement for development from egg to adult of 278. From these data, it has been calculated that *N. tenuis* would be able to complete a maximum of one full generation per year under typical UK summer conditions, with very little development possible between the months of October and April. Field trials carried out during the summer of 2008 supported these findings. Eggs placed in the field at the beginning of June and July hatched and the resulting nymphs completed development to adult in approximately 60 d. Eggs placed in the field at the beginning of August also hatched, but all the nymphs died at the beginning of November prior to moulting to fifth instar, apparently due to prolonged exposure to temperatures below the developmental threshold. Eggs placed in the field at the beginning of

September did not hatch (G. E. Hughes, unpublished data), possibly due to deleterious effects of night temperatures as opposed to the lack of thermal budget for development.

The results obtained in this study indicate that this strain of *N. tenuis* does not possess any diapause trait. Thus 100% of females reared under short day conditions (20°C, 12:12 LD) laid eggs immediately after transfer to a fresh plant and long day conditions (20°C, 16:8 LD). As a comparison, 73.3% of *Dicyphys hesperus* Knight (Hemiptera: Miridae), an omnivorous mirid also used for the control of glasshouse pests, reared from egg to adult under similar conditions (18°C, 12:12 LD) did not lay eggs on transfer to a 'summer regime' and were therefore considered to be in diapause (Hatherly *et al.*, 2008). Thus, although it is possible that diapause might be induced in *N. tenuis* under different temperatures or photoperiodic regimes to those used in this study, this seems unlikely, and it can be concluded that *N. tenuis* does not possess a diapause trait. The inability to diapause is an indication that *N. tenuis* may not be able to overwinter outside of the glasshouse environment.

The SCP of the different age groups and treatments of *N. tenuis* were all between -21.5° and -17.6°C, whereas the $LTemp_{50}$ ranged from -13.1° to -12.1°C with 100% mortality in all four treatments at -14.5°C or higher. The SCPs were similar to that of *D. hesperus* (Hatherly *et al.*, 2008); however, unlike this cold hardy mirid, 100% mortality was observed in *N. tenuis* at temperatures significantly above the SCP.

Lower lethal time experiments were carried out to investigate the survival of the mirids over longer time periods at temperatures more likely to be experienced in the field. The $LTime_{50}$ at 5°C ranged from 6.1 d for nymphs to 8.9 d for adults. Nymphs were significantly less cold hardy than adults, but there was no difference between the non-acclimated and acclimated groups. The duration of survival of *N. tenuis* was significantly less than that of *D. hesperus*, where the $LTime_{50}$ at 5°C of nymphs and adults occurred after 54 and 101.7 d respectively (Hatherly *et al.*, 2008).

A strong positive correlation between maximum field survival time and $LTime_{50}$ at 5°C in five non-native biological control agents was reported by Hatherly *et al.* (2005) and has been confirmed by subsequent investigations (Hatherly *et al.*, 2008; Allen, 2010). This allows potential non-native biocontrol agents intended for use in glasshouse biocontrol to be classified as high, medium or low risk in terms of their ability to establish outdoors in cool

temperate climates such as the UK. Based on its $LTime_{50}$ value at 5°C, it can be predicted from the relationship in Figure 3.6 that *N. tenuis* would die out in the field in less than a month; as predicted, 100% mortality occurred in all treatment groups within 23 d in the field, during a period of winter when temperatures did not fall below 0°C.

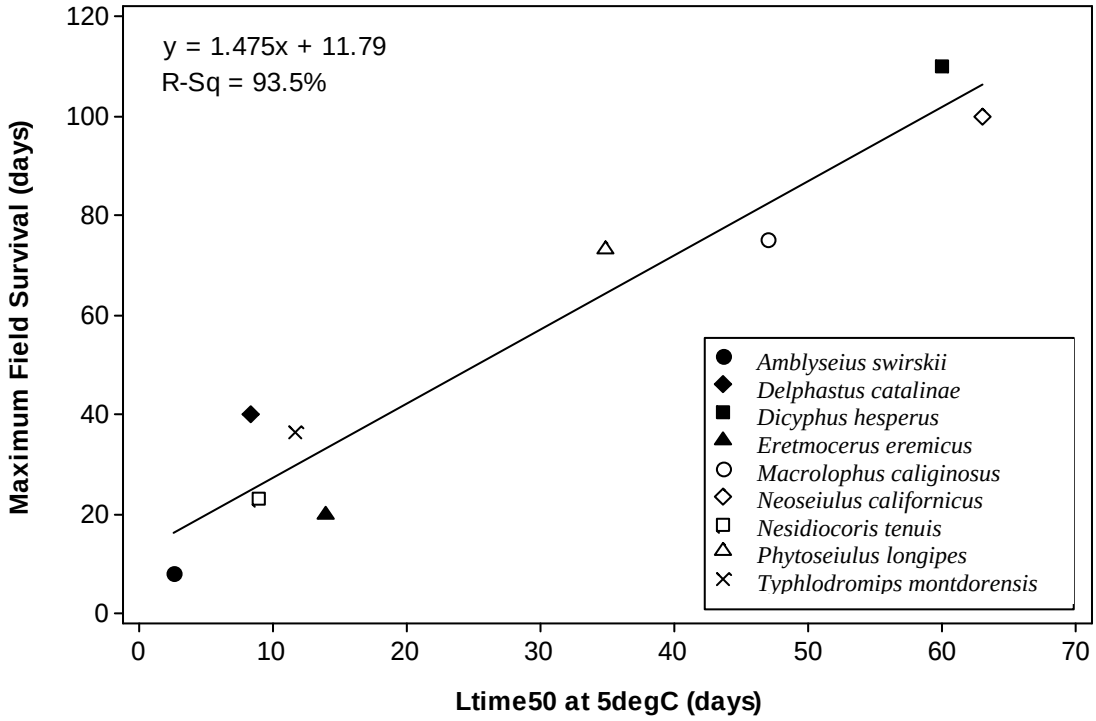


Figure 3.6: Relationship between the maximum field survival time and $LTime_{50}$ at 5°C for non-native invertebrate species in the UK. Sources of data: *Amblyseius swirskii* (Allen, 2010), *Delphastus catalinae* (Tullett, 2002), *Dicyphus hesperus* (Hatherly *et al.*, 2008), *Eretmocerus eremicus*, (Tullett *et al.*, 2004), *Macrolophus caliginosus* (Hart *et al.*, 2002b), *Neoseiulus californicus* (Hart, 2002a), *Nesidiocoris tenuis* (Hughes *et al.*, 2009), *Phytoseiulus longipes* (Allen, 2010), *Typhlodromips montdorensis* (Hatherly *et al.*, 2003).

Additionally, the inability of *N. tenuis* to undergo an acclimatory response following 7 d exposure to 10ºC is further indication of the species lack of cold tolerance. Similar results were achieved in an earlier field trial conducted between 23rd January and 8th February 2007 (G. E. Hughes, unpublished data) when temperatures briefly dropped below 0°C and 100% mortality was observed in all treatment groups within 22 d. This places *N. tenuis* in the 'low risk' category in terms of its ability to establish in temperate climates (Fig. 3.6), in marked

contrast to the related *M. caliginosus* (Hart *et al.*, 2002b) and *D. hesperus* (Hatherly *et al.*, 2008). The differences in establishment potential between these mirids are related, at least in part, to differences in their cold tolerance with *M. caliginosus* being more cold hardy than *N. tenuis* but not as cold hardy as *D. hesperus*. This is also reflected to a certain extent in their original distributions with *N. tenuis* and *M. caliginosus* being of Mediterranean origin and *D. hesperus* originating from North America and known to survive through Canadian winters (Gillespie & Sanchez, 2004). With respect to *N. tenuis*, the mirid lies in a group of control agents comprising predators and parasitoids with very limited cold tolerance (Figure 3.6).

In terms of the data requirements for non-native species for commercial release in the UK and other EU countries, the data obtained for *N. tenuis* indicate that this species lacks the level of cold tolerance that would be required to allow even a limited level of winter field survival outside of glasshouse environments in cool temperate or colder climates and is therefore likely to constitute a 'safe' biological control agent in such regions.

CHAPTER 4

Thermal activity thresholds of the predatory mirid *Nesidiocoris tenuis*: implications for its efficacy as a biological control agent.

4.1 ABSTRACT

This study investigates the thermal activity thresholds of the predatory mirid *Nesidiocoris tenuis* Reuter (Hemiptera: Miridae) and twospotted spider mite *Tetranychus urticae* Koch (Acari: Tetranychidae). Adult *N. tenuis* lost locomotory function and entered chill coma at significantly lower temperatures (4.0° and 0.3°C respectively) than adult *T. urticae* (7.0° and 5.7°C, respectively). However, the mirids were more adversely affected by high temperatures with *T. urticae* losing the ability to walk and entering heat coma at higher temperatures (47.3° and 49.7°C, respectively) than *N. tenuis* (43.5° and 46.6°C, respectively). Across a range of temperatures (2.5° to 20°C) adult *N. tenuis* had faster walking speeds than *T. urticae*. These data are discussed in relation to the climatic conditions under which *N. tenuis* would be an effective biocontrol agent.

4.2 INTRODUCTION

The possible impact of non-native biocontrol agents on native species is a subject of considerable current interest (van Lenteren *et al.,* 2006), as exemplified by studies on the Harlequin ladybird *Harmonia axyridis* (Pallas) (Coleoptera: Coccinellidae) (Majerus *et al.,* 2006; Brown *et al.,* 2008; Lombaert *et al.,* 2008; Poutsma *et al.,* 2008). Various reports have shown that winter low temperatures in northern Europe are an effective barrier to outdoor establishment for a number of non-native control agents (Tullett, 2002; Tullett *et al.,* 2004; Hatherly *et al.,* 2005; Hughes *et al.,* 2009); however, reports have also identified species such as *Neoseiulus californicus* McGregor (Acari: Phytoseiidae) where considerable cold tolerance and a previously unidentified diapause trait have enabled permanent outdoor establishment in

the UK (Jolly, 2000). Thus, the temperature–establishment interaction forms the first part of an integrated 'environmental risk assessment' for non-native biocontrol agents (van Lenteren *et al.*, 2006; van Lenteren & Loomans, 2006a,b).

Although much focus has been placed on temperature as a lethal constraint to establishment, it also impacts on other thresholds which can affect the distribution and, therefore, potential impact of a control agent. These thresholds include temperatures above or below which a species becomes incapable of development or activity. Whilst often overlooked in favour of the lethal effects of temperature as an indicator of establishment ability, in temperate regions where conditions are rarely severe enough to result in mortality directly, many species are unable to survive due to an inability to find food resources or breed successfully because of these sub-lethal constraints (Mellanby, 1939).

In the context of biological control, these species-specific thresholds can have a direct bearing on the efficacy of a control agent, as they are likely to influence the 'balance' in the interactions between predators and their prey, and parasitoids and their hosts. In light of this, it would seem advantageous for a control agent to be more active than its prey across a range of temperatures, and to remain active at temperatures above or below those which result in immobility of its prey. Thus, knowledge of these sub-lethal thermal tolerance traits will contribute to identification of successful control agents.

A number of techniques have been developed to investigate activity thresholds such as heat and chill coma. These methods often involve the observation of the 'righting response' of individual organisms (e.g. Renault *et al.*, 1999; Gilbert *et al.*, 2001; Castaneda *et al.*, 2004, 2005). This approach usually involves removing the subjects from a temperature-controlled environment (such as a water bath) at regular intervals, turning the individuals on to their dorsal surface and monitoring their ability to right themselves. As well as being laborious and subject to error, there has been concern that these methods result in disturbances which might affect both the body temperature and physiological status of the subjects and subsequently, the trait being observed (Hazell *et al.*, 2008). A new technique for studying activity thresholds was recently described by Hazell *et al.* (2008) in which multiple organisms can be cooled in a temperature-controlled arena with the behaviour of the organisms recorded by video capture technology. This technique allows distinct behaviours to be monitored without the disturbance of the organisms, and enables a more detailed

analysis of subtle movements such as the last twitch of an appendage, signifying entry into heat or chill coma.

This method was used in this study to analyse the various behavioural and physiological thresholds of *Nesidiocoris tenuis* Reuter (Hemiptera: Miridae) and a prey species *Tetranychus urticae* Koch (Acari: Tetranychidae). These data are presented and discussed with regard to the efficacy of *N. tenuis* as a predator, and more widely in terms of this approach as part of the process for identifying novel control agents.

4.3 MATERIALS AND METHODS

The materials and methods used for the identification of thermal activity thresholds in *N. tenuis* and a prey species *T. urticae* are described in chapter 2. The rearing methods of the mirids and mites are described in sections 2.2.1 and 2.2.2, respectively. The experimental system used is described in section 2.4.1 and the thermal thresholds investigated were as defined in section 2.4.2. Chill coma, chill coma recovery and heat coma experiments were carried out on 30 individuals from each species, age and treatment group (second to third instar nymphs and adult mirids and mites in non-acclimated and acclimated states) using methods described in sections 2.4.3, 2.4.4 and 2.4.5, respectively. The walking speeds of the treatment groups were analysed using the methods described in section 2.4.6. The statistical analysis used was as described in section 2.4.7.

4.4 RESULTS

4.4.1 CT_{min} and chill coma

As the temperature decreased, the organisms lost the ability to move their limbs in a coordinated manner and were, therefore, unable to walk. In all treatments, this thermal threshold (CT_{min}) occurred at temperatures above 0°C (Table 4.1). A further reduction in temperature resulted in entry into chill coma, the mean temperature of which was also above 0°C (Table 4.1).

Table 4.1: Mean (± SE) and range (in brackets) of temperatures (°C) at which non-acclimated and acclimated adult mites and non-acclimated and acclimated adult and nymphal *N.tenuis* reach their CT_{min} (T1), enter chill coma (T2) and undergo chill coma recovery (T3) and activity recovery (T4) (n = 30 for each treatment group).

Treatment	CT_{Min} (T1)	Chill coma (T2)	Chill coma recovery (T3)	Activity recovery (T4)
Non-acclimated adult mites	7.0 ± 0.3[d] (4.2 to 9.8)	5.7 ± 0.3[e] (3.1 to 8.4)	8.1 ± 0.3[e] (6.3 to 10.0)	8.9 ± 0.4[b] (6.8 to 11.2)
Acclimated adult mites	4.6 ± 0.1[c] (2.5 to 5.8)	3.9 ± 0.1[d] (2.4 to 5.4)	6.8 ± 0.4[d] (5.3 to 8.9)	7.8 ± 0.5[a] (5.6 to 11.4)
Non-acclimated adult mirids	4.0 ± 0.1[b] (2.7 to 4.8)	0.3 ± 0.1[a] (-1.0 to 1.7)	3.6 ± 0.3[b] (2.3 to 5.4)	9.7 ± 0.2[c] (8.7 to 10.8)
Acclimated adult mirids	3.3 ± 0.2[a] (1.7 to 5.3)	0.2 ± 0.1[a] (-1.4 to 1.9)	1.6 ± 0.3[a] (0.8 to 3.5)	8.5 ± 0.3[b] (7.3 to 9.9)
Non-acclimated nymphal mirids	8.2 ± 0.2[e] (5.6 to 9.6)	2.5 ± 0.1[c] (1.6 to4.2)	5.0 ± 0.3[c] (3.4 to7.0)	13.7 ± 0.6[e] (10.5 to16.9)
Acclimated nymphal mirids	4.7 ± 0.2[c] (3.0 to 6.5)	1.9 ± 0.1[b] (1.1 to 2.6)	4.8 ± 0.3[c] (3.3 to 6.8)	11.1 ± 0.5[d] (8.9 to 13.8)

Means followed by the same letters are not significantly different from one another (comparisons made using Bonferroni 95%CI).

Differences in the mean CT_{min} of *T. urticae* and adults and nymphs of *N. tenuis* were highly significant (χ^2=514.17, DF=5, P<0.001). Comparisons of the Bonferroni 95% confidence intervals indicated that non-acclimated and acclimated adult *N. tenuis* had a significantly lower CT_{min} (T1: ≤4.0°C) than the nymphal *N. tenuis* and mite treatments (T1: 4.6° to 8.2°C) and acclimation significantly decreased the CT_{min} of adult and nymphal *N. tenuis* and *T. urticae*. Further differences were identified in the shape and scale parameters of the samples (χ^2=46.56, DF=5, P<0.001), with non-acclimated mites showing the largest range in T1 values and non-acclimated adult mirids having the smallest range. The distribution ranges for all other treatment groups were similar (Table 4.1).

Significant differences were also observed for the temperatures at which the organisms entered chill coma (χ^2=710.37, DF=5, P<0.001) (Table 4.1). Exposure to the acclimation regime resulted in a lower chill coma temperature in nymphal mirids and mites; however, acclimation had no effect on the coma temperature of the adult mirids. Non-acclimated and

acclimated adult *N. tenuis* had the lowest chill coma temperatures (T2: 0.2°C and 0.3°C respectively). There were also significant differences in shape and scale parameters of the treatments (χ^2=41.38, DF=5, P<0.001), with the greatest variance occurring in the non-acclimated mite treatments (Table 4.1).

4.4.2 Chill coma recovery and activity recovery

As the temperature was increased from the chill coma temperature to the rearing or acclimation temperature, the organisms regained the ability to move their appendages and then walk. The mean temperature at which the individuals first entered chill coma recovery (T3) and activity recovery (T4) are shown in Table 4.1.

There were significant differences in the mean chill coma recovery temperatures (χ^2=959.54, DF=5, P<0.001). Exposure to the acclimation regime significantly reduced the chill coma recovery temperature of the adult mirids and mites but had no effect on the nymphal *N. tenuis* treatments. Both non-acclimated and acclimated adults and nymphs of *N. tenuis* began to recover at significantly lower temperatures than the mite treatment groups. There were no differences in the shape or scale parameters of the distributions of the treatments (χ^2=3.80, DF=5, P=0.58) (Table 4.1).

Significant differences were also observed in the temperatures at which the different experimental types regained the ability to walk (χ^2 = 328.81, DF = 5, P<0.001), with acclimated groups having lower activity recovery temperatures (T4) than non-acclimated groups. There were also differences between mirids and mites: both non-acclimated and acclimated nymphs of *N. tenuis* had relatively high activity recovery temperatures (T4: 11.1° and 13.7°C respectively), whilst adult mirid and mite treatments were able to regain locomotory function at temperatures of less than 9.7°C. The shape or scale parameters of the distributions were also different (χ^2=38.73, DF=5, P<0.001), with both treatment groups of adult mirids having the smallest range of values for T4 (Table 4.1).

4.4.3 CTmax and heat coma

There were differences in the temperatures at which the organisms lost the ability to walk (χ^2=1891.10, DF=5, P<0.001) (Table 4.2) with non-acclimated and acclimated mites having higher CT_{max} (T5: 48.0° and 47.3°C, respectively) than mirids of both age groups and treatments (T5: ≤43.5°C). The acclimation regime had no effect on the temperatures at which the adult mirids stopped walking, but resulted in a higher CT_{max} in the nymphal mirids and mites. Differences in the shape and scale parameters of the distributions were also evident (χ^2=30.92, DF=5, P<0.001), with non-acclimated and acclimated adult mirids having the greatest range of heat coma temperatures (Table 4.2).

Table 4.2: Mean (± SE) and range (in brackets) of temperatures (°C) at which non-acclimated and acclimated adult mites and non-acclimated and acclimated adult and nymphal *N. tenuis* lose the ability to walk (CT_{Max}) and enter heat coma (n=30 for each treatment group).

Treatment	CT_{Max} (T5)	Heat coma (T6)
Non-acclimated adult mites	47.3 ± 0.3 [d] (46.1 to 48.3)	49.7 ± 0.3 [d] (48.4 to 51.3)
Acclimated adult mites	48.0 ± 0.2 [e] (46.3 to 49.1)	50.2 ± 0.3 [d] (48.8 to 51.7)
Non-acclimated adult mirids	43.5 ± 0.4 [c] (41.5 to 46.0)	46.6 ± 0.3 [c] (45.7 to 48.2)
Acclimated adult mirids	43.1 ± 0.3 [c] (41.3 to 44.6)	45.1 ± 0.3 [b] (43.5 to 46.7)
Non-acclimated nymphal mirids	41.9 ± 0.2 [a] (40.8 to 43.1)	44.0 ± 0.2 [a] (42.9 to 45.2)
Acclimated nymphal mirids	42.6 ± 0.2 [b] (41.7 to 43.7)	44.7 ± 0.2 [b] (43.2 to 46.0)

Means within columns followed by the same letters are not significantly different (comparisons made using Bonferroni 95%CI).

Differences were observed for the temperatures at which the organisms entered heat coma (χ^2=2522.79, DF=5, P<0.001) (Table 4.2), with non-acclimated and acclimated mites having the highest values (T6: ≈ 50°C) compared with T6≤46.6°C for both mirid age groups. Whilst

comparisons of the Bonferroni 95% confidence intervals indicated that acclimation had a significant effect on the mean heat coma temperatures of the adult and nymphal mirids, the values differed by less than 1.5°C. Acclimation had no effect on the heat coma temperature of *T. urticae*. There were no differences in the shape or scale parameters of the experimental types (χ^2=2.96, DF=5, P=0.71) (Table 4.2).

4.4.5 Walking speed

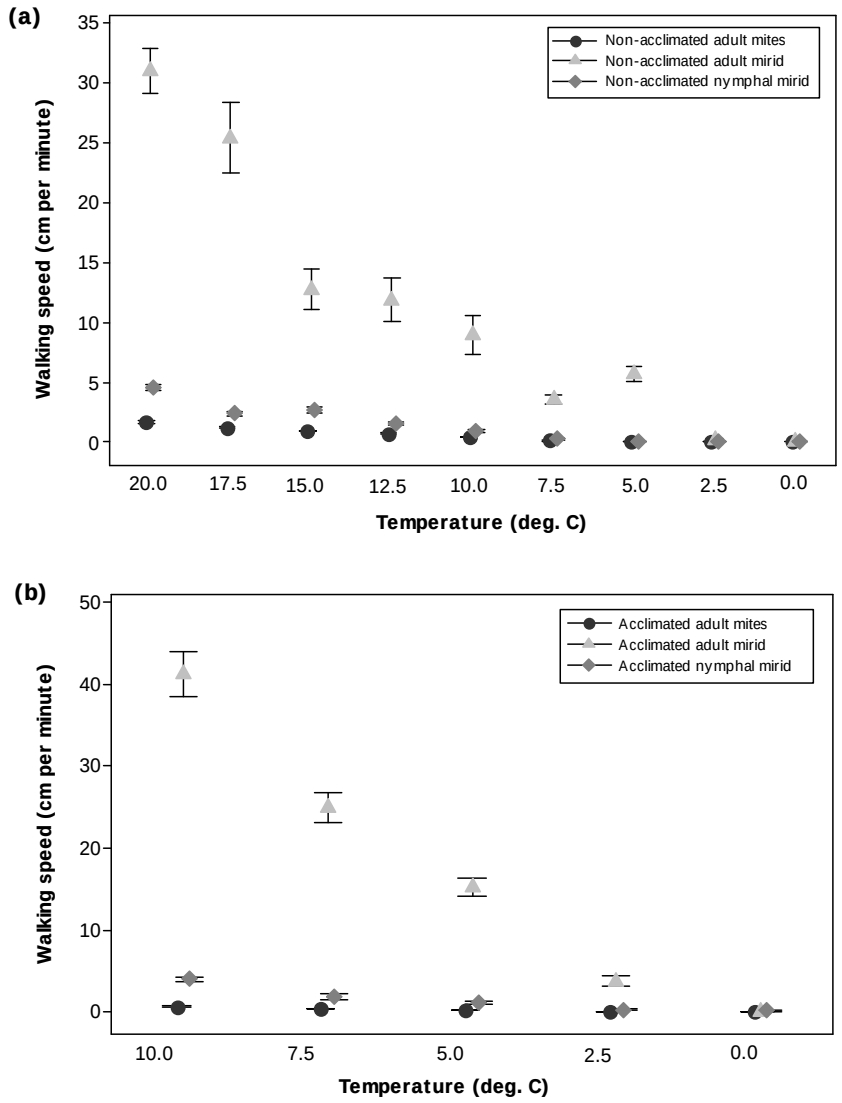


Figure 4.2. Mean walking speed (±1 SE) of (a) non-acclimated and (b) acclimated nymphal and adult *Nesidiocoris tenuis* and adult *Tetranychus urticae* at a range of constant temperatures (n=30 for each treatment group).

For non-acclimated treatments, Scheirer-Ray-Hare tests showed significant effects of both treatment ($H=205$, $DF=2$, $P<0.001$) and temperature ($H=43$, $DF=8$, $P<0.001$) on the walking speeds of the different experimental groups (Fig. 4.2a). The same was true for the effect of treatment ($H=114$, $DF=2$, $P<0.001$) and temperature ($H=279$, $DF=4$, $P<0.001$) on the acclimated organisms (Fig. 4.2b). Non-acclimated adult mirids were faster than mites at temperatures between 5° and 20°C ($P<0.001$), and non-acclimated nymphal mirids were faster than mites at temperatures between 10° and 20°C ($P<0.001$). For the acclimated treatments, adult mirids had faster walking speeds than mites at temperatures between 2.5° and 10°C ($P<0.001$) and nymphal mirids were faster than mites at temperatures between 5° and 10°C ($P<0.001$).

4.5 DISCUSSION

Most studies on the thermal biology of insects have focussed on strategies of overwintering and the lethal effects of low temperatures (Turnock & Fields, 2005; Pitts & Wall, 2006; Hatherly *et al.,* 2008). However, temperature has a direct effect on other species-specific thermal thresholds such as the ability to develop, walk or fly. Temperatures above or below these thresholds can reduce or prevent movement which could indirectly increase mortality because of an individuals' inability to find food resources or to evade predation or parasitism. With regard to biological control, the identification of these sub-lethal thermal tolerance traits can help to determine the potential efficacy of a control agent. For example, a predator would have a selective advantage over its prey if it had a lower developmental threshold, more generations per year, was more active across a range of temperatures, and remained active at temperatures above or below which its prey had become immobile. These processes and interactions have a major influence on the balance of natural predator-prey and host-parasitoid relationships, and are clearly important in the selection of biological control agents. The techniques used in this study to identify the sub-lethal thermal tolerance traits of *N. tenuis* and a prey species *T. urticae,* have a number of advantages over those used in the past (see Lutterschmidt & Hutchison, 1997; Renault *et al.,* 1999; Gilbert *et al.*, 2001). The apparatus described allows the simultaneous observation of multiple organisms without direct handling or disturbance of the individuals during the investigation and a permanent record of the experiment is captured, allowing retrospective analysis (and reanalysis) when required.

Also, the subtle movements that signify entry into chill coma can be identified. This could be important information if the temperature at which these events occurred caused some irreversible lethal or sub-lethal damage in some species.

Non-acclimated and acclimated adults of *N. tenuis* retained the ability to walk at temperatures below those which rendered the prey species *T. urticae* immobile. Conversely, the mirids were not as tolerant to heat and lost the ability to move in a coordinated manner at temperatures significantly below the upper thermal limits of *T. urticae*. These data indicate that in cool temperate climates such as those experienced in northern Europe, *N. tenuis* is likely to be an effective control agent against species with a similar activity profile to *T. urticae*, due to its ability to maintain locomotor function at comparatively low temperatures. However, in regions where temperatures may regularly approach 40°C, *N. tenuis* may not be as effective in the control of such pest species as it has a lower tolerance of high temperatures.

The relative walking speeds of *N. tenuis* and *T. urticae* also provide more information on the effectiveness of the predator. Across a range of temperatures (2.5° to 20°C), non-acclimated and acclimated adult and nymphal *N. tenuis* were faster than both treatment groups of *T. urticae*, suggesting that the mirids would be able to catch their spider mite prey quickly and efficiently.

To further assess the efficacy of candidate biological control agents, the study system could be extended to investigate the effect of other factors on their activity. For example, leaf surface morphology is considered to be important when assessing the activity of a control agent (De Clercq *et al.*, 2000; Koveos & Broufas, 2000; Cédola *et al.*, 2001; Skirvin & Fenlon, 2001) as movement be greatly affected by factors such as the presence of hairs or a waxy cuticle. Using leaf discs placed on the base of the arena, the video-capture set-up could be easily adapted to analyse the effects of different leaf substrates. Furthermore, investigations could be extended to include detailed observations of direct predator-prey interactions with the aim of predicting rates of predation under a range of environmental conditions.

With regard to the dispersal ability of the mirid, although acclimation increased the mobility of *N. tenuis* at lower temperatures, neither non-acclimated nor acclimated adult *N. tenuis* were able to fly when maintained in a controlled environment room at 10°C, nor in the

experimental arena at the same temperature, whereas this was common in mirids maintained in cages at 20°C and above. This suggests that the dispersal abilities of *N. tenuis* from autumn to spring in temperate regions such as the UK would be limited. In addition, Hughes *et al.* (2009) showed that *N. tenuis* lacks the cold tolerance to survive through winter in such climates. Therefore, whilst some dispersal from glasshouses would be possible in the summer, and assuming that as a polyphagous predator *N. tenuis* could successfully utilise native species as a food source, the mirid would at most have only a transient effect on the native ecosystem.

CHAPTER 5

Thermal biology and establishment potential in temperate climates of the parasitoid *Lysiphlebus testaceipes.*

5.1 ABSTRACT

Lysiphlebus testaceipes (Cresson) (Hymenoptera: Braconidae, Aphidiinae) is a parasitic wasp which plays an important role in the biological control of a number of aphid species. Through assessment of its thermal biology and low temperature tolerance, this study ascertains the establishment potential of *L. testaceipes* in cool temperate climates typical of northern Europe. The developmental threshold of *L. testaceipes* was 5.8°C. Rearing of parasitoids at shorter day lengths and lower temperatures indicated no ability to enter a diapause state. The SCP of non-acclimated and acclimated parasitoid life stages were between -24.6 and -17.7°C, with $LTemp_{50}$ temperatures approaching these values, indicating a high level of cold tolerance at short exposures. At 5°C the $LTime_{50}$ of acclimated larvae within parasitized aphids was 42.8 d; acclimated pupae continued to develop with 54% adult emergence from mummies within 60 d. Acclimated parasitoid larvae and pupae, within living and mummified aphids, continued to develop during 70 d of winter field exposure and emerging adult parasitoids were reproductively viable under field conditions. These data indicate that where suitable host species are available throughout the year, *L. testaceipes* would be able to establish in northern Europe.

5.2 INTRODUCTION

Biological control is considered to be a 'green' approach to pest management. However, in spite of the high number of releases and very few reports of associated negative effects (van Lenteren *et al*. 2006), over the last 20 years there has been an increasing trend towards the regulation of non-native biocontrol agents. In Australia and New Zealand, the guidelines are

so strict that very few alien introductions are permitted (van Lenteren *et al*. 2002) and legislation in Norway prevents the import of any species that cannot be proven to be native to the country (Hokkanen, 2003). In Europe however, the situation has been complicated by the lack of consistency across borders, with considerable variation between countries in the information that companies have to supply when submitting license requests for the import and release of non-native species. In countries that have some form of regulation, establishment potential forms a major part of the ERA and is, therefore, often the first aspect to be addressed (Hart *et al*., 2002a,b; Tullett *et al*., 2004; Hatherly *et al*., 2008; Hughes *et al*., 2009). This is especially true of the ERA of control agents to be released within glasshouses in temperate regions, the idea being that such species would pose little or no threat to the native ecosystem if it can be shown that climatic conditions would act as a barrier to the establishment of escaping organisms.

Lysiphlebus testaceipes (Cresson) (Hymenoptera: Braconidae, Aphidiinae), is an important biological control agent for a broad range of aphid species (Starý *et al*., 1988a, 2004; Silva *et al*., 2008), including the economically important pest of cereal crops, Schizaphis graminum (Rondani) (Jackson *et al*., 1970; Rodrigues & Bueno, 2001). This parasitoid, originally introduced to southern France from Cuba in the 1970s, spread rapidly over Mediterranean France, Italy and Spain (Starý *et al*., 1988a,b). Although currently available as a commercial control agent in these regions, *L. testaceipes* has become a species of renewed interest because of the decision of the joint panel of EPPO (European and Mediterranean Plant Protection Organisation) and IOBC (International Organisation for Biological and Integrated Control of Noxious Animals and Plant-Western Palaearctic Regional Service) to remove it from the EPPO 'positive list' of so called 'safe species' (List of biological control agents widely used in the EPPO region) at the panel meeting in 2008. Factors that led to this decision included a consideration of its wide host range (Starý *et al*., 1988a,b, 2004; Silva *et al*., 2008), expanding geographic distribution (Starý *et al*., 2004), and its ability to adapt to and thrive in a range of environments from agro-ecosystems to forests (Starý *et al*., 1988a,b), characteristics which ultimately led to the displacement of native parasitoids (Tremblay, 1984; Starý *et al*., 1988b, 2004). However, in spite of the apparent risks associated with the use of this species as a control agent in southern Europe, under certain conditions the likelihood of such negative effects could be more limited. For example, as this parasitoid has not previously been released in the UK and other northern European countries, if it was shown to lack the cold tolerance necessary for outdoor establishment in these climates, the

species could have potential as a glasshouse biocontrol agent. Whilst it is known that *L. testaceipes* occurs in areas in the USA with cold winters (Jones *et al*., 2008), there appears to be regionally and genetically distinct populations that differ in cold hardiness (Shufran *et al*., 2004).

Against this background, the work described in this chapter investigates the cold tolerance of *L. testaceipes* as part of an assessment of its establishment potential in cool temperate climates that occur throughout northern Europe. In a wider context, the data will be used to examine the relationship between laboratory survival at 5°C and winter field survival as part of the ERA of non-native biocontrol agents (Hatherly *et al.*, 2005, 2008; Hughes *et al.*, 2009).

5.3 MATERIALS AND METHODS

5.3.1 Rearing of Lysiphlebus testaceipes

The rearing methods of *L. testaceipes* are described in chapter 2, section 2.2.3.

5.3.2 Effect of temperature on rate of development

The effect of temperature on development was determined using a method similar to that described by Hatherly *et al*. (2008) and Hughes *et al*. (2009) in studies on non-native species with potential as biocontrol agents in Europe. Six adult parasitoids were placed in each of 48 Blackman boxes containing a *V. faba* leaf infested with 10 second or third instar aphids at 23ºC, 16:8 LD. The parasitoids were left on the plants for 4 h to mate and parasitize the aphids. Following the removal of the parasitoids, six Blackman boxes were placed in each of eight incubators at 9.5, 13, 15, 18, 19.5, 21, 25 and 28ºC, 16:8 LD. The plants were monitored daily and the number of days post oviposition corresponding with mummy formation, was recorded. As the mummies developed, they were removed from the leaf and placed individually into secure, ventilated glass vials, on a 1 cm layer of agar (2%) (Oxoid Ltd, UK) covered with a circular disk of filter paper to act as a moisture source to prevent the desiccation of the parasitoid pupae. The vials were checked daily and time taken for adult

emergence was recorded. The number of emerging parasitoids at each temperature varied from 32 to 51, reflecting both the variance in the rate of parasitism and the survival of the parasitoid life stages at different temperatures. The results were analyzed using simple and weighted linear regression. The line was weighted using the inverse of the variance (Draper & Smith, 1981), overcoming any problems of variance heterogeneity and resulting in greater emphasis being placed on values at lower temperatures (Hart *et al*., 2002b; Olsen *et al*., 2003). The developmental threshold was estimated by identifying the temperature at which the weighted linear regression line crossed the x-axis (Hart *et al*., 2002b; Olsen *et al*., 2003).

The results were analysed using the method described in section 2.3.1.

5.3.3 Diapause

Six adult parasitoids were placed on aphid infested *V. faba* leaves (10 aphids per leaf) within 24 Blackman boxes for 4 h to mate and lay eggs at 23ºC, 16:8 LD. Following removal of the parasitoids, the Blackman boxes were transferred to short day conditions (8:16 LD), 12 at 9.5 ºC and 12 at 15ºC. The parasitized aphids were maintained under these conditions until adult parasitoids emerged. The time taken from oviposition to emergence was recorded. The resulting adults were placed in new Blackman boxes on fresh *V. faba* leaves, infested with 10 aphids. Depending on the number of emerging parasitoids per day, three to five parasitoids were placed in each Blackman box, ensuring that there was at least one female and one male in each. Half the boxes from each temperature were maintained under the same conditions for 4 h and the other half were transferred to long-day conditions (23ºC, 16:8 LD) for the same time period. After 4 h the parasitoids were removed and all of the Blackman boxes were transferred to long-day conditions (16:8 LD) at 23ºC. The leaves were then monitored daily for the formation of mummies and emergence of parasitoids.

5.3.4 Supercooling points

Experiments were carried out as described in section 2.3.3 on six treatment groups: larvae within parasitized aphids, pupae within mummified aphids and adult parasitoids, all in non-acclimated and acclimated states. The sample size for each treatment group varied from 39 to 41.

5.3.5 Lower lethal temperature

The lower lethal temperature (LTemp) experiments were carried out as described in chapter 2, section 2.3.4, on the same six treatment groups as for the SCP experiment (chapter 5, section 5.3.4).

Following exposure, adult parasitoids from the treated and control groups were maintained at 10ºC, 16:8 LD, in a ventilated glass vial containing a fresh cotton wool wick saturated in a honey and water solution. Mortality was assessed 72 h after exposure. This temperature was selected following preliminary investigations indicating that adult longevity was reduced at higher temperatures. Parasitoids were included in the mortality count if they were unable to walk in a coordinated manner or right themselves. For parasitoids exposed as pupae within mummified aphids (again from both treated and control groups), the samples were placed in fresh vials at 23ºC, 16:8 LD, on a 1 cm layer of agar (2%) and adult emergence was monitored. If adult parasitoids failed to fully emerge within 3 weeks, the organisms were included in the mortality count. For parasitoids exposed in the larval stage within living aphids, individuals from both the treated and control groups were placed on fresh *V. faba* leaves within Blackman boxes (10 per box) at 23ºC, 16:8 LD. If the parasitized aphids failed to develop through to mummies and yield adult parasitoids within 4 weeks, the organisms were included in the mortality count.

5.3.6 Lower lethal times

Lower lethal time experiments were carried out on the same six treatment groups as for SCP and lethal temperature experiments. For each adult treatment group (non-acclimated and acclimated), 10 individuals were placed in a ventilated glass vial containing a cotton wool wick steeped in a honey and water solution. Parasitoids within mummified aphids (non-acclimated and acclimated) were also placed in glass vials (10 per vial) on a 1 cm layer of agar (2%), covered with a circular piece of filter paper to prevent desiccation. Non-acclimated and acclimated parasitoid larvae, within living aphids, were placed on *V. faba* leaves within Blackman boxes. Again, 10 organisms were placed in each box. For each treatment group and at each temperature, 75 vials or Blackman boxes were set up. The lethal time experiment was then carried out as described in chapter 2, section 2.3.5.

A control sample of 50 of each parasitoid life stage was set up as described for the treatment groups. Adult parasitoids were held at 10ºC for the duration of the experiment with mortality assessed at 3 d intervals. Mortality in the control larval and pupal stages was assessed by following the development of the samples at 23ºC, 16:8 LD. Individuals that failed to develop into mummies and yield adult parasitoids were included in the mortality count.

Treatment groups exposed at 5ºC, and control groups, were transferred into fresh vials or Blackman boxes at 5 to 6 d intervals to refresh the food source and prevent the formation of mould spores that could result in increased mortality in the samples. At -5 and 0ºC, the treatment groups did not need to be transferred due to the shorter period of exposure and the lower temperatures preventing the formation of fungal spores.

5.3.7 Field mortality

The field experiments were conducted from 19th November 2009 to 27th January 2010. The treatment groups (non-acclimated and acclimated larvae, pupae and adult parasitoids) were set-up as for the LTime experiments (60 – 70 vials or Blackman boxes per treatment), with the addition of non-acclimated and acclimated unfed adult samples. These unfed treatments were also placed in ventilated glass vials, but unlike the fed adults, the cotton wool wick was steeped only in water. The Blackman boxes and vials were placed in secure, ventilated containers in the field in a sheltered location at the University of Birmingham, UK. A Tinytalk® datalogger (Gemini, UK), placed within each box, recorded the temperature throughout the experiment. Plastic trays (45 × 35 cm) were placed on top of each box to provide protection from direct sunlight.

For each treatment, samples (five replicates of 10) were taken from the field at regular intervals, placed at 10ºC for 1 h to prevent mortality due to heat shock, and mortality was recorded using the same methods and criteria as for the LTemp and LTime experiments. Again, as for the LTime experiments, precautions were taken for the duration of the field trial to prevent the formation of mould spores and to replenish food sources (see section 5.3.6 above).

In addition to assessing mortality at regular intervals, the viability of adults emerging from mummies in the field was tested. Adult parasitoids were collected on days 25, 35 and 45

after placing in the field and placed in Blackman boxes containing *V. faba* leaves infested with 10 second to third instar *A. fabae.* Five to seven parasitoids were placed in each of one to four Blackman boxes (depending on the number of parasitoids found at each sampling interval and in each treatment group) in the field, ensuring that there were at least two males and two females per box. To allow time for mating and oviposition, parasitoids were removed from the Blackman boxes after 3 d. These parasitoids were then transferred into fresh Blackman boxes containing *A. fabae* on *V. faba* leaves, within a 10ºC incubator. Again, parasitoids were left for 3 d to mate and parasitize the aphids. Following removal of the parasitoids, Blackman boxes from the field and 10ºC incubator were placed at 23ºC, 16:8 LD and monitored daily for the formation of mummies and emergence of adult parasitoids. Aphids were exposed to adult parasitoids at 10ºC to assess the reproductive viability of parasitoids under conditions known to favour oviposition (as indicated by preliminary experiments), and in the field, to determine the ability of the parasitoids to locate and parasitize a host under potentially deleterious winter field conditions.

A control sample of 50 of each parasitoid life stage was set up and mortality assessed as described for the LTime experiments (see section 5.3.6).

5.3.8 Host-range of Lysiphlebus testaceipes

Whilst *L. testaceipes* is known to be a successful parasitoid of *A. fabae*, this aphid rarely overwinters as a mobile stage in the UK, with eggs typically hatching late February to early March. Thus, in the event that *L. testaceipes* is able overwinter in the UK, they could not rely on *A. fabae* as a host species during this time. Nevertheless, *L. testaceipes* is known to have an extensive host range (Mackauer & Starý, 1967; Starý *et al.*, 1988, 2004; Silva *et al.*, 2008). Therefore, as part of the ERA of this particular population, the ability of the parasitoid to parasitize aphids known to overwinter in the UK as mobile stages (anholocyclic clones) was investigated. The four species examined were the bird cherry–oat aphid, *Rhopalosiphum padi* L. (Hemiptera: Aphididae), the peach-potato aphid, *Myzus persicae* (Sulzer) (Hemiptera: Aphididae), the grain aphid, *Sitobion avenae* F. (Hemiptera: Aphididae) and the cabbage aphid, *Brevicoryne brassicae* L. (Hemiptera: Aphididae). Furthermore, the percentage parasitism of these species was compared with that of the black bean aphid, *Aphis fabae*, to determine the relative acceptance of each host.

To investigate the parasitism rates of *B. brassicae* and *M. persicae*, 50 second or third instar aphids of each species were placed on Chinese cabbage leaves, (*Brassica campestris* L. ssp. *pekinensis*), within Blackman boxes. Ten aphids were placed on each leaf, allowing for five samples of 10 per species. Six parasitoids were placed in each box and left for 4 h to allow time for mating and oviposition. The samples were monitored over 4 weeks and the formation of mummies and adult emergence was recorded.

The parasitism rates of *R. padi* and *S. avenae* were investigated in the same way. Fifty second instar aphids from each species were placed on five separate blades of wheat, *Triticum aestivum* L., within Austin tubes (Austin *et al*., 1991) (10 aphids per blade of wheat). Again, 6 parasitoids were placed in each of the tubes for 4 h to allow time for mating and oviposition. Following removal of the parasitoids, the samples were monitored daily to record formation of mummies and the emergence of adults.

5.3.9 Statistics

The SCP results were analysed using a Kruskal-Wallis test and individual groups were compared using an unequal variance two-sample t-test (see Ruxton, 2006), where the alpha level was set at 0.05.

The lower lethal time, lower lethal temperature and field mortality data were analysed using methods described in chapter 2, section 2.3.8.

Differences in the rates of parasitism of *L. testaceipes* on *A. fabae, B. brassicae, M. persicae, R. padi* and *S. avenae* were identified using a KruskalWallis test.

5.4 RESULTS

5.4.1 Developmental threshold

The mean number of days for development of the parasitoid from egg to mummy, from mummy to adult and from egg to adult is shown in Table 5.1. Developmental time for each life stage decreased with increasing temperature.

Table 5.1: Effect of rearing temperature on the developmental time (mean days ± SE) of *Lysiphlebus testaceipes* (16:8 LD).

Temp. (°C)	N	Egg - mummy	Mummy - adult	Egg to adult
9.5	36	28.5 ± 0.5	21.6 ± 0.2	49.9 ± 0.5
13.0	32	15.1 ± 0.3	9.5 ± 0.2	24.6 ± 0.4
15.0	41	13.2 ± 0.4	7.2 ± 0.2	20.4 ± 0.3
18.0	51	9.0 ± 0.1	5.6 ± 0.2	14.7 ± 0.2
20.0	45	8.6 ±0.2	7.8 ± 0.1	13.4 ± 0.2
22.0	47	7.3 ± 0.1	4.5 ± 0.2	11.8 ± 0.3
25.0	49	6.5 ± 0.2	3.6 ± 0.2	10.1 ± 0.2
28.0	42	5.5 ± 0.2	3.2 ± 0.1	8.7 ± 0.2

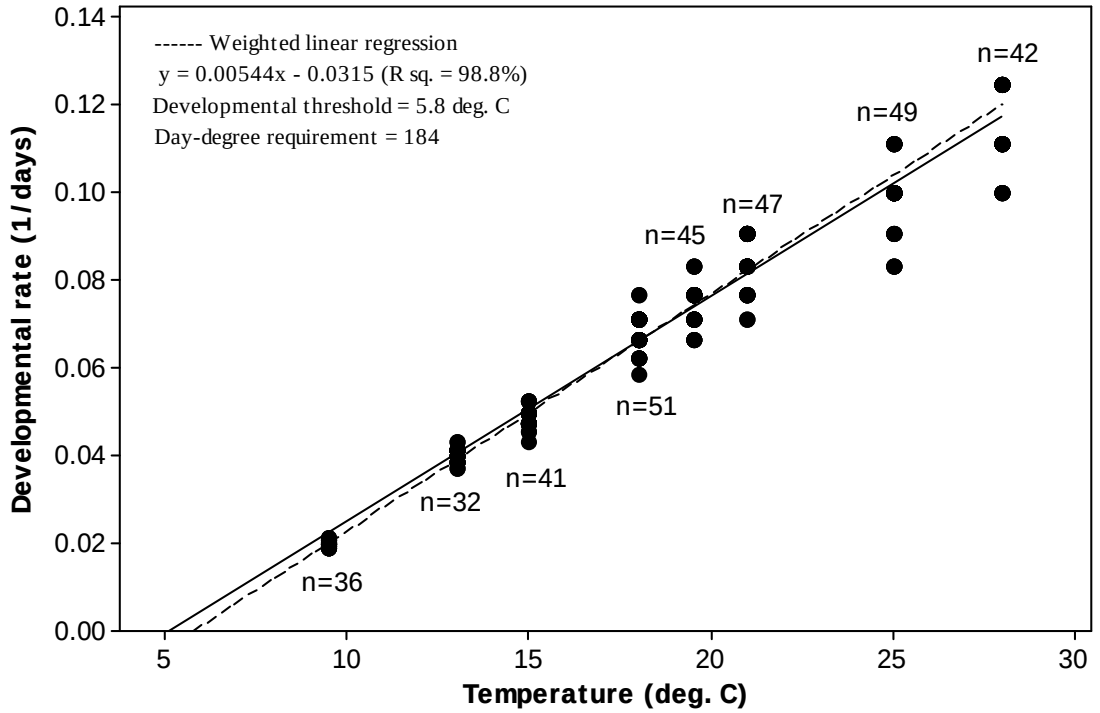


Figure 5.1: Developmental rate of *Lysiphlebus testaceipes* from egg to adult at eight constant temperatures under long-day conditions (16:8 LD). Lines fitted by simple linear regression (solid line) and weighted linear regression (broken line). Developmental threshold determined by extrapolation of the fitted line (weighted linear regression) to the x-axis.

The developmental threshold, derived from the weighted linear regression, was estimated to be 5.8°C (Fig. 5.1), with a day degree requirement for development from egg to adult of 184. The threshold for emergence of adults from mummified aphids (calculated in the same way as the developmental threshold) was estimated to be 5.5ºC (Fig. 5.2). Across all temperatures, newly emerged adults were observed to mate and lay eggs within 24 h of emergence.

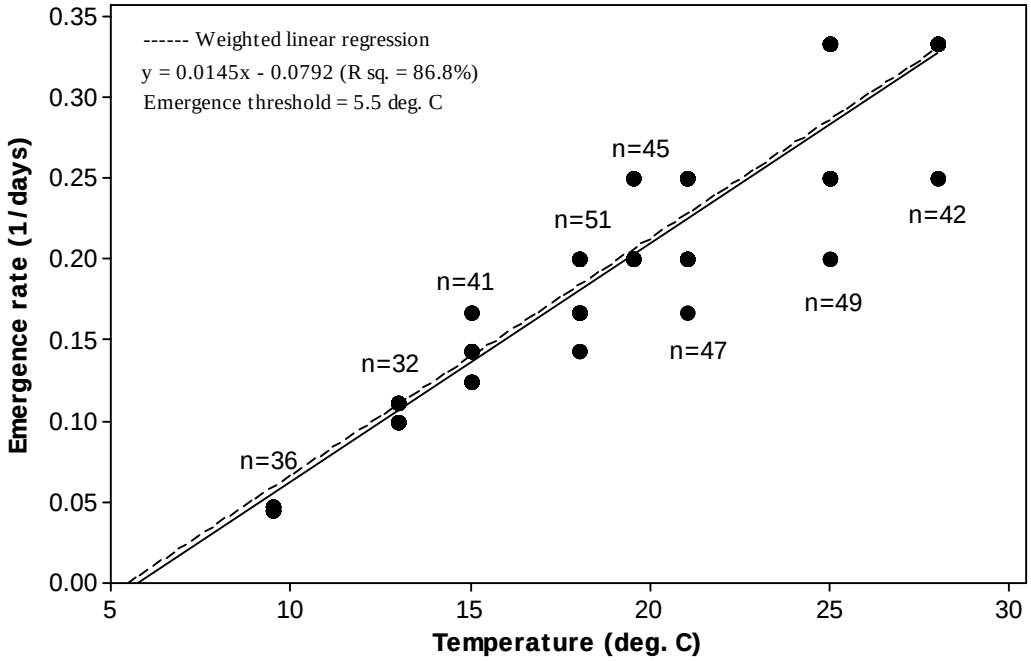


Figure 5.2: Emergence rate of adult *Lysiphlebus testaceipes* from mummies at eight constant temperatures under long-day conditions (16:8 LD). Lines fitted by simple linear regression (solid line) and weighted linear regression (broken line). Emergence threshold determined by extrapolation of the fitted line (weighted linear regression) to the x-axis.

To determine the likely annual voltinism of *L. testaceipes* in the UK over the past 15 years, the available thermal budget was calculated by subtracting the developmental threshold from the mean daily temperature for each day, and then summing these values to obtain an annual total. With knowledge of the day degree requirement per generation, these data were then used to estimate the annual voltinism under outdoor conditions (Table 5.2).

Table 5.2: Estimated annual voltinism of *Lysiphlebus testaceipes* over 15 years in Birmingham UK.

Year	Annual available day degrees	Theoretical number of generations per year
1993	1530	8.3
1994	1746	9.5
1995	1941	10.5
1996	1584	8.6
1997	1853	10.1
1998	1751	9.5
1999	1849	10.0
2000	1747	9.5
2001	1769	9.6
2002	1815	9.9
2003	1867	10.1
2004	1893	10.3
2005	1834	10.0
2006	2030	11.0
2007	1761	9.6
Mean	**1798**	**9.8**

Table 5.3: Estimated voltinism (cumulative day degrees [DD] in brackets) of *Lysiphlebus testaceipes* by season over 15 years in Birmingham, UK.

Year	Spring (March -May)	Summer (June -Aug.)	Autumn (Sept. - Nov.)	Winter (Dec. -Feb.)
1993	1.8 (326)	4.6 (838)	1.6 (288)	0.4 (78)
1994	1.5 (284)	5.2 (949)	2.4 (437)	0.4 (76)
1995	1.7 (313)	5.5 (1017)	2.7 (501)	0.6 (110)
1996	1.1 (202)	5.0 (923)	2.3 (425)	0.2 (34)
1997	1.9 (350)	5.3 (983)	2.4 (448)	0.4 (72)
1998	2.0 (363)	4.6 (851)	2.3 (422)	0.6 (115)
1999	2.1 (392)	4.9 (903)	2.6 (477)	0.4 (77)
2000	1.7 (316)	4.9 (905)	2.3 (428)	0.5 (98)
2001	1.6 (290)	5.1 (934)	2.8 (509)	0.2 (36)
2002	1.9 (343)	4.9 (908)	2.4 (435)	0.7 (129)
2003	1.9 (347)	5.6 (1037)	2.3 (415)	0.4 (68)
2004	1.9 (357)	5.3 (982)	2.5 (458)	0.5 (96)
2005	1.8 (327)	5.1 (933)	2.8 (516)	0.3 (58)
2006	1.8 (331)	5.8 (1065)	3.1 (565)	0.4 (69)
2007	2.1 (384)	4.7 (862)	2.4 (433)	0.5 (82)
Mean	**1.8 (328)**	**5.1 (939)**	**2.5 (450)**	**0.4 (80)**

It can be seen that over a 15 year period (1993 to 2007), *L. testaceipes* would be capable of an average of 9.8 generations per year. Further analyses of the data indicate that some development would be possible throughout winter (December to February) (Table 5.3), with mean daily temperatures regularly exceeding the developmental threshold. Parasitoids surviving this period could then complete development and reproduce in spring (March – April) when higher temperatures allow an average of 1.8 generations to occur.

5.4.2 Diapause

The mean developmental time of *L. testaceipes* reared under short-day conditions (8:16 LD), at 15°C was 20.4 ± 0.1 d (Fig. 5.3). This was similar to estimates obtained using simple and weighted linear regression equations derived from the developmental experiments (19.7 and 20.0 d, respectively). It was also the same as the actual developmental time of *L. testaceipes* under long day conditions (16:8 LD), 20.4 ± 0.1 d (Fig. 5.1). A one-way ANOVA confirmed that day length (8:16 or 16:8 LD) had no effect on the developmental rate of *L. testaceipes* at 15°C (ANOVA: $F_{1,72}$ = 0.24, P = 0.62). Additionally, emerging adults successfully parasitized second to third instar *A. fabae* within 24 h of emergence.

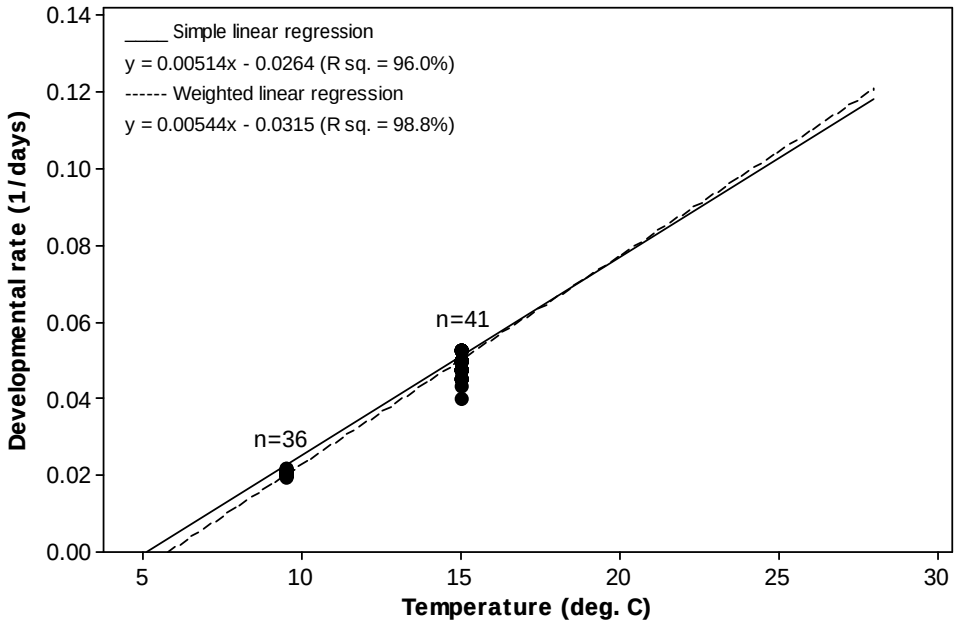


Figure 5.3: Developmental rate of *Lysiphlebus testaceipes* at 9.5°C and 15°C under short-day conditions (8:16 LD), superimposed onto fitted lines from Figure 5.1 (other data points omitted for clarity).

At 9.5°C, 8:16 LD, the average developmental time of the parasitoids from egg to adult was 49.3 d. Estimates for developmental time at 9.5ºC under a long-day light regime (16:8 LD), using simple and weighted linear regression were 44.6 and 49.6 d, respectively. Again, the developmental times of parasitoids reared under these short-day conditions (9.5°C, 8:16 LD) were comparable to those of parasitoids reared at 9.5°C 16:8 LD (Fig. 5.3). There was no difference in the developmental times of parasitoids reared under these different light regimes (ANOVA: $F_{1,60}$ = 2.03; P = 0.16). Furthermore, adult parasitoids reared under these conditions were again able to mate and successfully parasitize aphids within 24 h of emergence.

5.4.3 Supercooling points

The mean SCP of the six treatment groups differed significantly (Table 5.4), with acclimated adult *L. testaceipes* having the highest SCP (-17.7°C) and non-acclimated pupae within mummies the lowest (-24.6°C). Post-hoc two-sample t-tests showed that acclimation reduced the SCP of parasitized aphids (t=-3.27, DF=60, P=0.002) but resulted in an increase in the SCP of mummies (t=3.09, DF=72, P=0.003) and adult *L. testaceipes* (t=15.59, DF=74, P<0.001). These data indicate that, whilst parasitized aphids (and, therefore, the parasitoid larvae within them) are able to acclimate to lower temperatures, pupae and adult parasitoids show no acclimatory ability.

Table 5.4: Mean (±1 SE) and range of SCPs of non-acclimated and acclimated parasitized aphids and mummies and adult *Lysiphlebus testaceipes.*

Experimental group	N	Mean ± SE (°C)	Range (°C)
Non-acclimated parasitized aphid	40	-23.1 ± 0.2	-26.0 to -16.5
Acclimated parasitized aphid	40	-24.4 ± 0.2	-26.3 to -22.2
Non-acclimated mummies	40	-24.6 ± 0.1	-26.0 to -23.1
Acclimated mummies	39	-24.0 ± 0.2	-26.4 to -22.0
Non-acclimated adult *L. testaceipes*	41	-23.5 ± 0.2	-25.9 to -19.4
Acclimated adult *L. testaceipes*	40	-17.7 ± 0.2	-21.8 to -14.7

5.4.4 Lower lethal temperatures

The temperatures resulting in 10, 50 and 90% mortality ($LTemp_{10, 50, 90}$) in the six treatment groups are shown in Figure 5.4. In the control groups, 2% mortality was recorded for adult parasitoids and 0% for parasitized aphids and mummies.

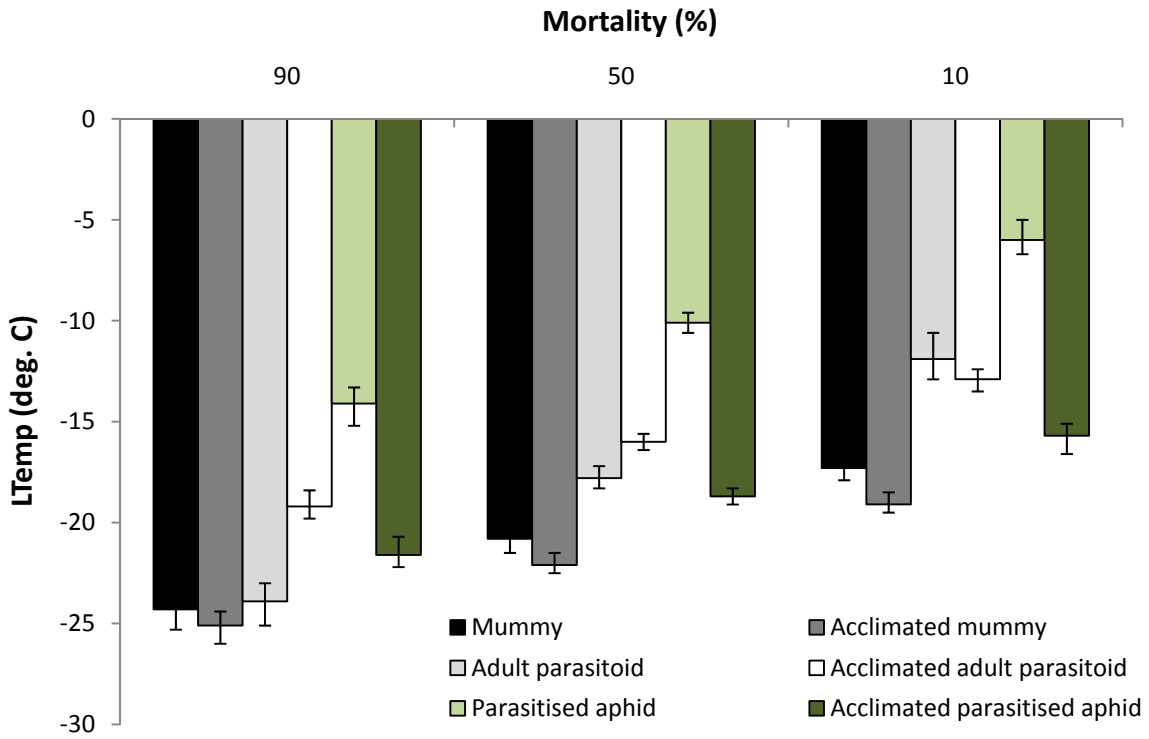


Figure 5.4: $LTemp_{10, 50}$ and $_{90}$ (±95% fiducial limits) of non-acclimated and acclimated larval, pupae and adult *Lysiphlebus testaceipes*.

There was no difference in the temperature at which 50% mortality occurred in non-acclimated and acclimated mummies (-22.8 and -22.1°C, respectively) (as indicated by overlapping fiducial limits), but there were differences in the temperatures corresponding to 50% mortality in both non-acclimated and acclimated adult parasitoids (-17.8 and -16°C, respectively) and non-acclimated and acclimated parasitized aphids (-10.1 and -18.7 °C, respectively). Thus, as for the SCP experiments, the parasitoids themselves showed no acclimatory response, whereas exposure to the acclimation regime significantly reduced the mortality of parasitoid larvae.

For the parasitized aphid treatment groups, 100% mortality occurred at temperatures significantly above their mean SCP. Conversely, the temperatures which resulted in 90% mortality in adult and mummy *L. testaceipes* were similar to that of their SCPs, indicating a high level of cold tolerance in short exposures.

5.4.5 Lower lethal times

The lower lethal times for the six treatment groups (non-acclimated and acclimated larvae, pupae and adult *L. testaceipes*) at -5, 0 and 5°C for 10, 50 and 90% ($LTime_{10, 50, 90}$) mortality, are shown in Figure 5.5a, b and c, respectively (with the exception of $LTime_{90}$ values for the pupal treatments at 5°C). Over the 25 d, there was 10% mortality of the adult control group and no mortality in the larval or pupal control groups.

Adult parasitoids did not show any acclimatory response, with exposure to 10ºC for 7 d reducing the $LTime_{50}$ of adult parasitoids at -5 and 5°C and having no effect on the $LTime_{50}$ at 0°C. However, exposure to the acclimation regime significantly increased the survival of both larval and pupal parasitoids across all three temperatures (as indicated by non-overlapping fiducial limits).

Across the three exposure temperatures (-5, 0 and 5°C), parasitoids exposed as pupae were significantly more cold tolerant than the other treatment groups. Furthermore, both non-acclimated and acclimated pupae continued to develop at 5°C, resulting in the emergence of adult parasitoids at this temperature (Fig. 5.6a and b). As a result of this continued development, after 60 d at 5°C, 36% of the non-acclimated mummies and 54% of the acclimated mummies had yielded parasitoids, making it unrealistic to give an $LTime_{90}$ value for these treatment groups. However, the total emergence decreased with time, showing that prolonged exposure to 5°C resulted in some mortality. These results are consistent with estimates of the temperature threshold for adult emergence from mummies (Fig. 5.2). With an emergence threshold of 5.5°C, it is reasonable to expect some emergence at 5°C and conversely, some mortality as a result of prolonged exposure to temperatures below this threshold.

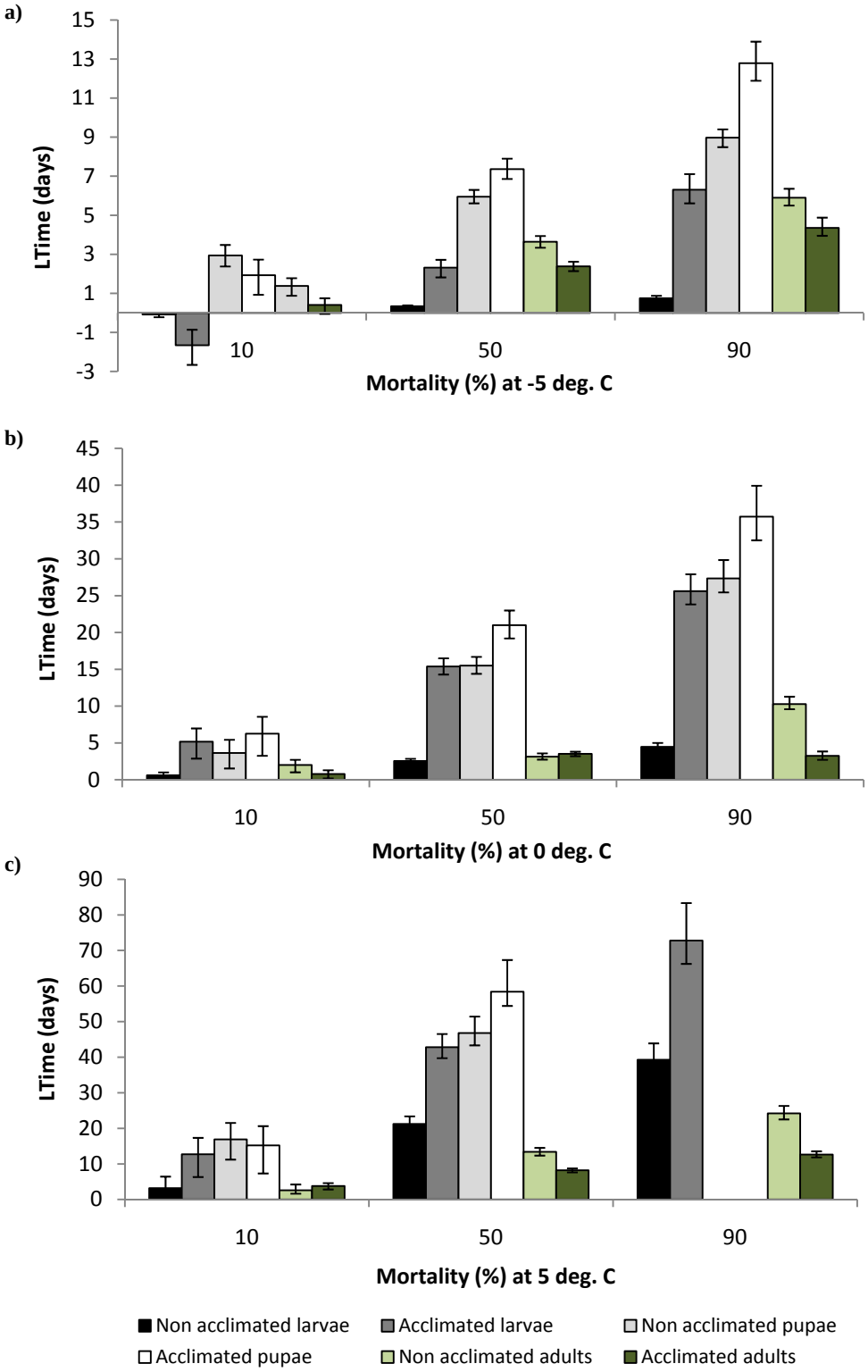


Figure 5.5: LTime $_{0,50}$ and $_{90}$ (± 95% fiducial limits) at (a) -5°, (b) 0° and (c) 5°C for non-acclimated and acclimated larval, pupae and adult *Lysiphlebus testaceipes.*

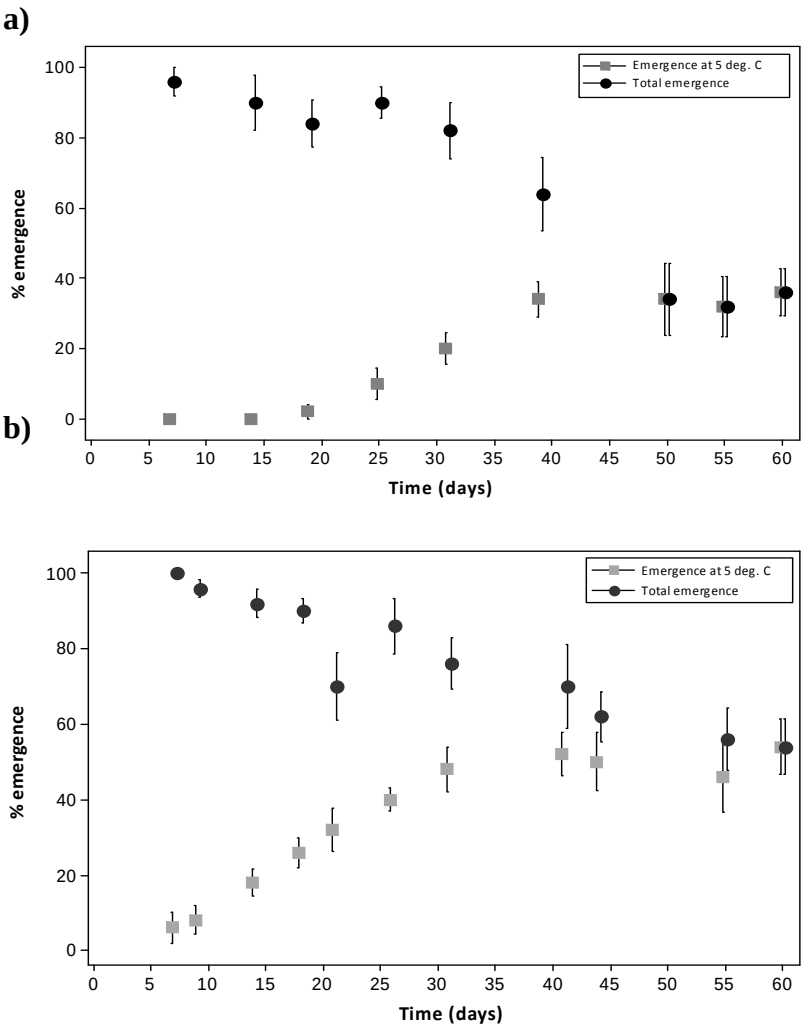


Figure 5.6: The % emergence of adult *Lysiphlebus testaceipes* from a) acclimated mummies and b) non-acclimated mummies at 5°C, both within the incubator and total emergence following removal from the incubator (n = 50 for each sample).

5.4.6 Field mortality

The mortality rates of larval and adult parasitoid treatments in the field from the 19th November 2009 to the 27th January 2010 are shown in Figures 5.7 and 5.8, respectively. For pupal treatments, percentage adult emergence is shown rather than mortality (Fig. 5.9). This is because, rather than dying in the mummified state, many individuals continued to develop in the field resulting in the emergence of adult parasitoids over the period of exposure (19th November to 27th January). The corresponding field temperatures are shown in Figure 5.10. Over the duration of the adult parasitoid field trial (35 d), 48% mortality was recorded in the adult control groups whereas only 2% mortality was recorded for the pupal and larval control

groups. This indicates that a relatively high proportion of the mortality observed for adult parasitoid treatments in the field could be attributed to ageing rather than wholly due to cold exposure.

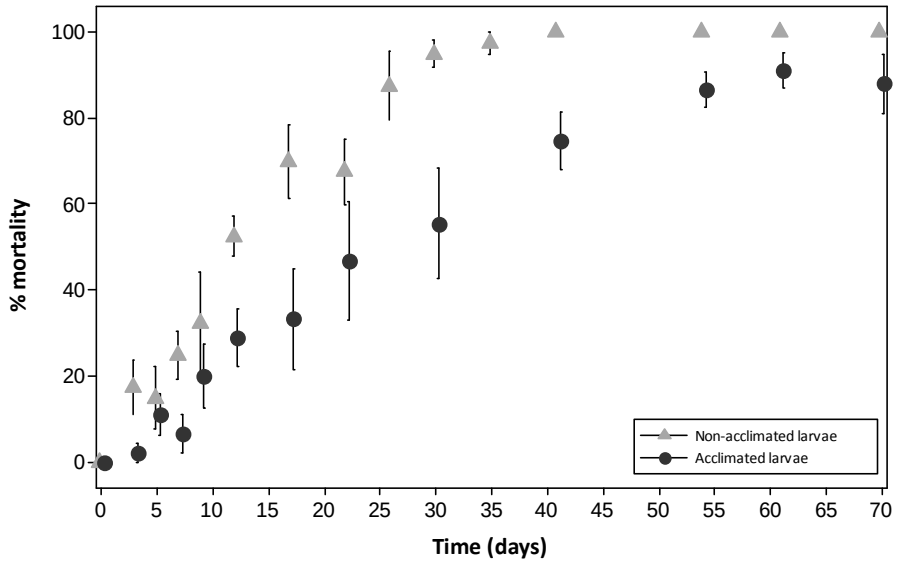


Figure 5.7: Mean mortality (±1 SE) of non-acclimated and acclimated larval *Lysiphlebus testaceipes* in the field from 19th November 2009 to 27th January 2010 (n = 50 for each sample).

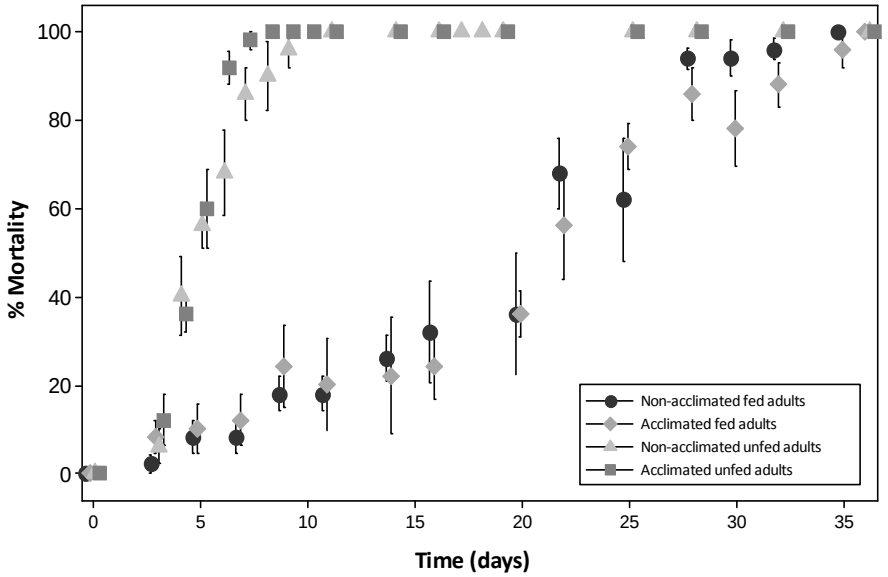


Figure 5.8: Mean mortality (±1 SE) of non-acclimated and acclimated adult *Lysiphlebus testaceipes* in the field with and without food from 19th November 2009 to 27th January 2010 (n = 50 for each sample).

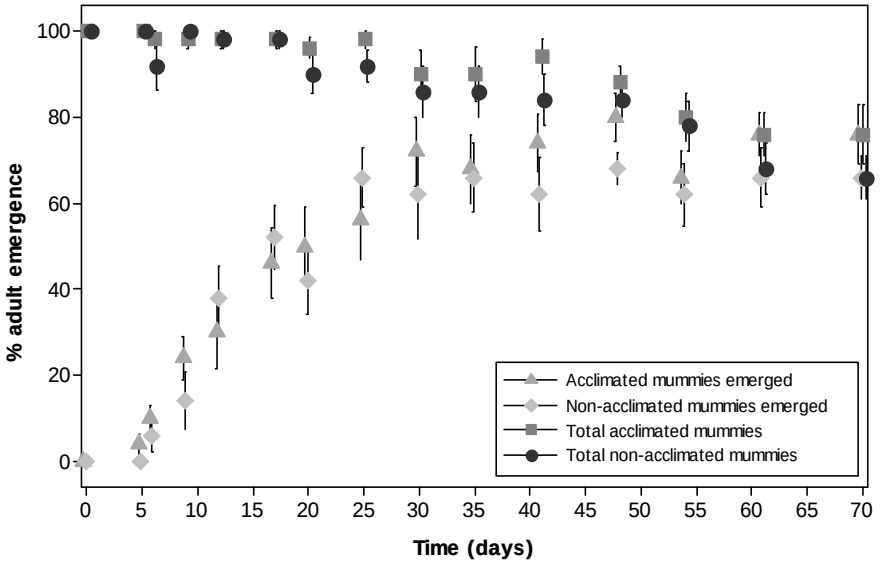


Figure 5.9: The emergence of adult *Lysiphlebus testaceipes* (± SE) from non-acclimated and acclimated mummies both within the field from 19th November 2009 to 27th January 2010and total emergence following removal from the field (n = 50 for each sample).

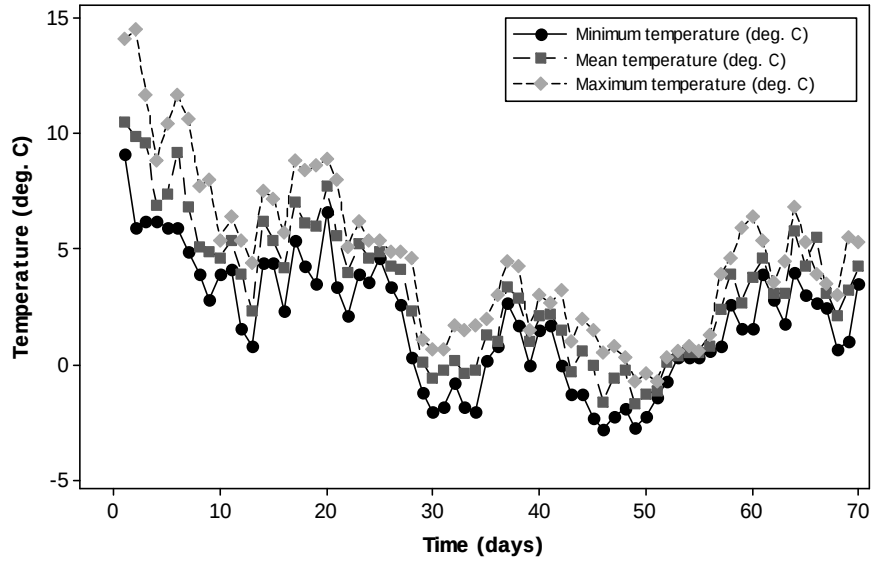


Figure 5.10: Minimum, maximum and mean temperatures experienced by non-acclimated and acclimated *Lysiphlebus testaceipes* life stages in the field from 19th November 2009 to 27th January 2010.

The averaged daily minimum, mean and maximum temperatures during the field trial were 1.8, 3.1 and 4.5°C respectively, with temperatures regularly falling below 0°C (Fig. 5.7). In

spite of these low temperatures, *L. testaceipes* still showed some development throughout the field trial, with adult parasitoids emerging from mummies in the field until day 61 and 100% of the surviving acclimated parasitized aphids developing into mummies by day 22.

As is evident from Figure 5.10, fed adults had a significantly lower mortality in the field than unfed adults (Z=-12.08, DF=1; P<0.001), with exposure to the acclimation regime further reducing survival in unfed adults (Z=-2.50, DF=1; P=0.01), but having no effect on mortality of fed animals (Z=0.69, DF=1; P=0.49). In larvae within parasitized aphids (Fig. 5.8), acclimated individuals were able to survive in the field for considerably longer than their non-acclimated counterparts (Z=4.9, DF=1; P<0.001), with surviving acclimated aphids developing into mummies in the field. For parasitoids placed in the field in the mummy stage, there were significant differences in the total emergence of adults (Z=2.49, DF=1; P=0.01), with a greater number of adults emerging from acclimated treatments. However, there was no difference in the emergence of adults from mummies in the field (Z=1.60, DF=1; P=0.11). Further to this, adults emerging from mummies in the field were shown to be reproductively viable. On days 25 and 35, emerging adults were able to parasitize aphids in the field whilst adults collected on day 45 were unable to do the same as a result of low temperatures affecting mobility (mean temperatures ≤ 0ºC). They were capable of successful parasitism when transferred to a temperature-controlled room at 10ºC.

5.4.7 Host range

Table 5.5: Mean parasitism rates (± SE) of *Lysiphlebus testaceipes* on 5 species of aphid, *Aphis fabae*, *Brevicoryne brassicae*, *Myzus persicae*, *Rhopalosiphum padi* and *Sitobion avenae*, with positive Z-values indicating a mean rank greater than the mean rank for all the observations, and a negative Z-value indicating a lower mean rank.

Species	N	Mean ± SE	Z-values	P-value
A. fabae	49	47.9 ± 1.3	3.40	< 0.001
B. brassicae	28	0.0 ± 0.0	-2.21	< 0.001
M. persicae	48	3.0 ± 2.0	-1.19	< 0.001
R. padi	45	24.9 ± 3.3	1.70	< 0.001
S. avenae	46	1.1 ± 1.1	-1.70	< 0.001

Significant differences occurred between the parasitism rates of *L. testaceipes* in *A. fabae*, *B. brassicae*, *M. persicae*, *R. padi* and *S. avenae* (H=18.88; DF=4; P<0.001) (Table 5.5), with *L. testaceipes* having the highest rate of parasitism in *A. fabae* (Z = 3.40) and the lowest rate in *B. brassicae* (Z = -2.21), with none of the exposed *B. brassicae* aphids being successfully parasitized.

5.5 DISCUSSION

Whilst the establishment potential of insects, mites and related invertebrates can be affected by a number of factors, in cold or temperate climates, establishment is often dependent on the ability of the species to tolerate winter low temperatures and to continue vital processes such as development and reproduction. As a result, this chapter focuses on the thermal biology and cold tolerance of *L. testaceipes* through a series of laboratory and field trials.

The developmental threshold of *L. testaceipes* was 5.8ºC with a day degree requirement from egg to adult of 184. From these data it was estimated that *L. testaceipes* would be able to complete an average 9.8 generations per year (calculated using temperature data from 1993-2007). The data obtained for the developmental threshold of *L. testaceipes* in this study were comparable to those of an earlier investigation (Royer *et al*., 2001), where the developmental responses of three geographic isolates of this parasitoid were examined. The colonies collected from southern Texas, central Oklahoma and central Nebraska had developmental thresholds of 5.6, 6.6 and 6.4 ºC, respectively. Whilst adult survival at 10ºC was greater in the Nebraskan geographic isolate, no differences in the developmental thresholds were observed amongst the wasp colonies.

Further examination of the temperature data indicates that the parasitoid would be capable of some development throughout winter (Table 5.3), with mean daily temperatures often being above the developmental threshold. This assumption was supported by winter field trials, where parasitoids continued to develop, with adults emerging from mummies in the field, and larvae within parasitized aphids developing into pupae and forming mummified aphids. These data also suggest that *L. testaceipes* does not possess a diapause trait, as a reduced rate of development or a delay in adult emergence from the mummies would have been expected

as an indication of diapausing ability (Mehrnejad & Copland, 2005). Furthermore, the parasitoids were shown to be capable of parasitism within 24 h of emergence from a diapause-inducing regime. Again, a delay in reproductive maturity would be expected in diapausing insects. The temperature and light regimes used in this experiment (15°C, 8:16 LD and 9.5°C, 8:16 LD) were similar to those that induced diapause (78.7%) in the parasitic wasp *Microplitis mediator* Haliday (Hymenoptera: Braconidae) (16°C, 8:16 LD) (Li *et al.*, 2008). Thus, although it is possible that diapause might be induced in *L. testaceipes* under different temperatures or photoperiodic regimes from those used in my study, it seems unlikely that *L. testaceipes* possesses a diapause trait.

The SCP of the six treatment groups (non-acclimated and acclimated parasitized aphids, mummies and adult parasitoids) were all between -17.7 and -24.6°C, whilst the $LTemp_{50}$ -values ranged from -10.1 for parasitized aphids to -22.1 for acclimated mummies. Therefore, whilst a small percentage of the parasitoid life stages survived temperatures close to their SCP (as evidenced by $LTemp_{90}$ values), the majority exhibited some pre-freeze mortality, though not to the same extent as other non-native biocontrol agents recently investigated (Tullett, 2002; Hatherly *et al.*, 2003; Tullett *et al.*, 2004; Hughes *et al.*, 2009; Allen 2010). In a similar experiment, investigating the SCPs of *L. testaceipes*, Jones *et al.* (2008) identified a difference between male and female *L. testaceipes* and discovered an inverse relationship between the age of the adult parasitioids and the point at which they spontaneously froze. For younger adult female parasitoids (<6 h after emergence), the SCP was less than -26°C (Jones *et al.*, 2008). Whilst the values obtained for this study were considerably higher (mean of -23.5 and -17.7°C for non-acclimated and acclimate parasitoids, respectively), the data are not directly comparable as the sexes were not separated in these experiments and adult parasitoids were used within 24h of emergence, with no distinctions made at shorter time intervals. Nevertheless, with a range of -25.9 to -19.4°C for non-acclimated adult *L. testaceipes*, it appears that the colony used during the investigations outlined in this thesis had a lower SCP to that used by Jones *et al.* (2008). Whilst this could be due to differences in the origin of the populations used, it is also possible that differences in the rearing methods led to a change in cold tolerance.

Lower lethal time experiments were carried out to investigate the survival of the parasitoid life stages over longer time periods, at temperatures more likely to be experienced in the field. Across the three temperatures (-5, 0 and 5°C), parasitoids exposed as pupae within

mummified aphids were significantly more cold tolerant than the other treatment groups. Moreover, both non-acclimated and acclimated pupae continued to develop at 5°C, resulting in the emergence of adult parasitoids at this temperature. As well as providing an indication of cold tolerance, these results may be important in informing producers as to the optimum storage times and conditions for parasitoid mummies. For example, if the parasitoids were stored in the pupal stage at 5°C, storage times would have to be limited, both to reduce mortality as a result of cold exposure and to limit parasitoid emergence.

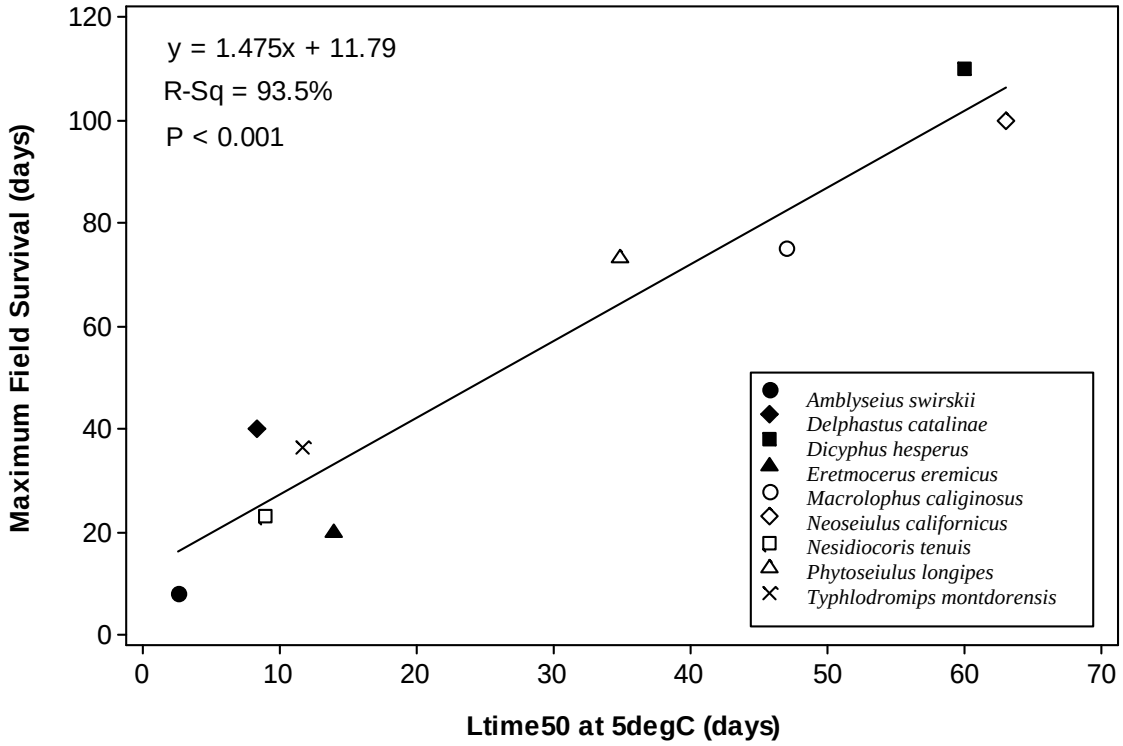


Figure 5.11: Relationship between $LTime_{50}$ at 5°C and the maximum field survival time for non-native invertebrate species in the UK. Sources of data: *Amblyseius swirskii* (Allen, 2010), *Delphastus catalinae* (Tullett, 2002), *Dicyphus hesperus* (Hatherly *et al.*, 2008), *Eretmocerus eremicus*, (Tullett *et al.*, 2004), *Macrolophus caliginosus* (Hart *et al.*, 2002b), *Neoseiulus californicus* (Hart, 2002a), *Nesidiocoris tenuis*, *Phytoseiulus longipes* (Allen, 2010), *Typhlodromips montdorensis* (Hatherly *et al.*, 2003).

The $LTime_{50}$ results were also used to further examine the relationship between laboratory survival at 5°C ($LTime_{50}$) and winter field survival (Hatherly *et al.*, 2005). A strong positive correlation between these two indices of cold tolerance was reported by Hatherly *et al.* (2005) in five non-native biocontrol agents, and confirmed by subsequent investigations with other candidate species (Hatherly *et al.*, 2008; Hughes *et al.*, 2009; Allen, 2010) (Fig. 5.11). This

correlation allows non-native species to be classified as high, medium or low risk in terms of their ability to establish outdoors in cool temperate climates such as the UK. To date, all species for which this assessment of establishment potential has been applied, died out quickly within 1 to 6 weeks (*Amblyseius swirskii, Delphastus catalinae* (Tullett, 2002), *Eretmocerus eremicus* (Tullett *et al.,* 2004), *Nesidiocoris tenuis* (Hughes *et al.,* 2009) and *Typhlodromips montdorensis* (Hatherly *et al.,* 2004)), survived for over 10 weeks whilst remaining in the same life cycle stage (*Phytoseiulus longipes* (Allen, 2010)), or developed slowly through sequential stages of their life cycle, with a small proportion surviving until spring (*Dicyphus hesperus* (Hatherly *et al.,* 2008), *Macrolophus caliginosus* (Hart *et al.,* 2002b) and *Neoseiulus californicus* (Hart *et al.,* 2002a)). *Lysiphlebus testaceipes*, however, having a lower developmental threshold that allowed continued development and emergence of reproductively viable adult parasitoids from mummies in the field in winter, falls into a different category from all these species. Unlike the other species, this level of cold tolerance was not immediately evident from analysis of the field mortality data, as no one individual life-stage was able to survive a whole winter. Nevertheless, as *L. testaceipes* was shown to be capable of parasitism in the field, it would not be necessary for individuals escaping from a glasshouse to survive until the following spring, as the population would be maintained by their progeny. For this reason, whilst *L. testaceipes* does not 'fit' anywhere in the correlative relationship between laboratory and field survival (Fig. 5.11), it is nevertheless a cold hardy species based on a combination of relatively long-lived larval, pupal and adult stages, and its ability to locate and parasitize aphid hosts in winter.

As the maintenance of an outdoor population of *L. testaceipes* in the UK is dependent on reproduction overwinter, it is also reliant on the availability of a suitable host species and ambient temperatures favouring movement, and thus host location, over the winter period. As adult parasitoids survived for up to 5 weeks in the field, it is highly likely that they would experience some periods of favourable conditions during this time. This assumption is substantiated by an analysis of temperatures during the field trial (19th November 2009 to 27th January 2010), in conjunction with data from another study investigating the movement thresholds of *L. testaceipes* (G. Hughes, unpublished data). Investigations into temperatures which resulted in the immobility of *L. testaceipes* indicated that the parasitoids lost the ability to walk at temperatures around 0°C (G. Hughes, unpublished data). As temperatures during the course of the field trial were regularly above 0°C, in spite of the winter being the coldest

on record since 1978-79 (Met Office UK, 2010), it is likely that the parasitoid would have had the capacity for dispersal and host location. In addition to this, *L. testaceipes* is known to have an extensive host range of over 100 aphid species (Mackauer & Starý, 1967, Starý *et al.*, 1988, Silva *et al.*, 2008), increasing the likelihood of a successful encounter during this time. Furthermore, investigations into this particular population have confirmed the presence of suitable host species (*M. persicae, R. padi* and *S. avenae*) that are common in the UK and known to overwinter in active stages.

In terms of the data requirements for the commercial release of non-native species in the UK and other EU countries, with the different life stages showing developmental ability throughout winter, and adult parasitoids capable of parasitism in the field, it is clear that during a milder winter, temperatures would not act as a barrier to establishment. These conclusions, together with knowledge of the extensive host range of *L. testaceipes*, indicate that this species would not constitute a 'safe' biological control agent in temperate regions.

CHAPTER 6

Thermal activity thresholds of the parasitic wasp *Lysiphlebus testaceipes*: implications for its efficacy as a biological control agent.

6.1 ABSTRACT

This chapter investigates the thermal activity thresholds of the parasitic wasp *Lysiphlebus testaceipes* (Cresson) (Hymenoptera: Braconidae, Aphidiinae) and a host species, the black bean aphid *Aphis fabae* Scop. (Hemiptera: Aphididae). Adult parasitoids lost locomotory function and entered chill coma at significantly lower temperatures (-0.1 and -8.0°C, respectively) than second instar *A. fabae* (5.6 and 2.3°C, respectively). Parasitoids were also more heat tolerant with CT_{max} (41.4°C) and heat coma temperatures (44.1°C) higher than those of the aphid (39.1 and 43.0°C, respectively). Furthermore, across a range of temperatures (0° to 20°C), *L. testaceipes* had considerably faster walking speeds than *A. fabae*. These data are discussed in relation to the climatic conditions under which *L. testaceipes* would be an effective control agent, and the likelihood of establishment and spread in northern European climates.

6.2 INTRODUCTION

Temperature has often been regarded as the main barrier to establishment for exotic species in an alien environment (Andrewartha & Birch, 1954; Bale & Walters, 2001; Bale, 2002; van Lenteren *et al*., 2006). This is especially true of ectothermic organisms, such as insects and mites, because of their limited ability to regulate body temperature (Bale & Hayward, 2010). For exotic invertebrates introduced into cooler climates, many studies have focussed on the lethal effects of winter conditions as a major constraint on establishment (Tullett, 2002; Tullett *et al.,* 2004; Hatherly *et al.,* 2005; Hughes *et al.,* 2009). Temperature also impacts on other important physiological processes such as an animal's ability to develop, reproduce and

move. In temperate regions, where conditions are rarely severe enough to cause direct mortality, sub-lethal temperatures which leave an insect incapable of activity or development can have a marked effect on species distribution (Bale, 2002) and indirectly lead to mortality through the inability of the insect to find food resources or breed successfully.

Such knowledge is particularly important when considering the risks associated with the release of non-native biocontrol agents. As escapes from glasshouses are unavoidable, it is useful to be able to predict the fate of individuals or populations leaving the confines of these environments. Through identification of certain thermal tolerance traits, it is possible to estimate the potential range and ease of movement of a species when conditions are favourable, thereby determining the likelihood that a species may establish and disperse (Ward & Masters, 2007). Traits commonly investigated include non-lethal measures such as heat and chill coma temperatures (Mellanby, 1939; Colhoun, 1960; Gaston & Chown, 1999), chill coma recovery (Ayrinhac *et al*., 2004; Castaneda *et al*., 2005) and walking speed under a range of temperatures (Hughes *et al*., 2010). These traits, as well as providing an insight into the ability of a control agent to tolerate and recover rapidly from exposure to temperatures close to their physiological thresholds, can also be used to determine the likely efficacy of the species under a range of conditions. For example, when identifying a successful candidate control agent, it would be advantageous for the predator or parasitoid to be more active than its prey across a range of temperatures, and to remain active under conditions that render the prey immobile (Hughes *et al*., 2010).

In this study, a technique developed by Hazell *et al.*, (2008) was used to identify these behavioural and physiological thresholds in the parasitic wasp *Lysiphlebus testaceipes* (Cresson) (Hymenoptera: Braconidae, Aphidiinae) and one of its host species, *Aphis fabae* Scop. (Hemiptera: Aphididae). Findings are presented and discussed with regard to the efficacy of *L. testaceipes* as a control agent of aphids, its thermal thresholds and movement at low temperatures, thereby providing information on its potential impact and spread should establishment occur.

6.3 MATERIALS AND METHODS

The materials and methods used for the identification of thermal activity thresholds in *L. testaceipes* and a prey species *A. fabae* are described in chapter 2. The rearing methods of the parasitoids and aphids are described in sections 2.2.2 and 2.2.3 respectively. The experimental system is described in section 2.4.1 and the thermal thresholds investigated were as defined in section 2.4.2. Chill coma and chill coma recovery experiments were carried out on 30 to 40 individuals from each species, age and treatment group (larval, pupal and adult parasitoids and aphids in non-acclimated and acclimated states) using methods described in sections 2.4.3 and 2.4.4. Heat coma experiments were carried out on 30 to 36 individuals from each treatment group using the method described in section 2.4.5. The walking speed of the treatment groups was analysed using methods described in section 2.4.6. The statistical analysis was as described in section 2.4.6.

6.4 RESULTS

6.4.1 CT_{min} and chill coma

As the temperature decreased, the organisms lost the ability to move their limbs in a coordinated manner and were therefore unable to walk (CT_{min}). A further reduction in temperature resulted in entry into chill coma. The temperatures at which these thermal thresholds occurred are shown in Table 6.1.

Differences in the mean CT_{min} of *L. testaceipes* and second instar *A. fabae* were highly significant (χ^2=1861.26; DF=3, P<0.001). Comparisons of the Bonferroni 95% confidence intervals indicated that non-acclimated and acclimated *L. testaceipes* had a significantly lower CT_{min} ($\leq$ -0.1°C) than both treatment groups of *A. fabae* ($\geq$ 3.9°C) and that acclimation significantly decreased the CT_{min} in both species (Table 6.1). Further differences were identified in the shape and scale parameters of the samples (χ^2=30.24; DF=3, P<0.001), with acclimated *A. fabae* showing the largest range in CT_{min} values. The distribution ranges for all other treatment groups were similar.

Significant differences were also observed for the temperatures at which the organisms entered chill coma (χ^2=8474.02, DF=3, P<0.001) (Table 6.1). The chill coma temperatures of the *L. testaceipes* treatment groups were below 0°C (≤ -8.0°C), whereas the mean chill coma temperatures of *A. fabae* were significantly higher at 0.5°C and above. Once again, exposure to the acclimation regime resulted in a lower chill coma temperature in the parasitoids and aphids. There were also differences in the shape and scale parameters of the treatments (χ^2=31.23, DF=3, P<0.001), with the greatest varience occurring in the aphid treatment groups.

Table 6.1: Mean (± SE) and range (in brackets) of temperatures at which non-acclimated and acclimated adult *Lysiphlebus testaceipes* and non-acclimated and acclimated second instar *A. fabae* reach their CT_{min}, enter chill coma, chill coma recovery and activity recovery (n = 30 to 40 for each treatment group).

Treatment	CT_{min} (°C)	Chill coma (°C)	Chill coma recovery (°C)	Activity recovery (°C)
Non-acclimated adult parasitoids	-0.1 ± 0.1[b] (-1.5 to 1.6)	-8.0 ± 0.1[b] (-9.0 to -6.3)	-5.2 ± 0.2[b] (-8.1 to -0.2)	6.1 ± 0.2[b] (4.8 to 9.2)
Acclimated adult parasitoids	-1.5 ± 0.1[a] (-3.0 to -0.4)	-9.9 ± 0.1[a] (-10.8 to -8.8)	-7.3 ± 0.2[a] (-10.7 to -5.8)	5.0 ± 0.1[a] (4.2 to 7.5)
Non-acclimated second instar aphids	5.6 ± 0.2[d] (3.3 to 6.8)	2.3 ± 0.2[d] (-2.0 to 4.3)	8.6 ± 0.2[d] (4.8 to 11.9)	12.3 ± 0.4[d] (7.4 to 18.1)
Acclimated second instar aphids	3.9 ± 0.2[c] (0.8 to 6.5)	0.5 ± 0.2[c] (-0.8 to 5.1)	7.5 ± 0.1[c] (6.2 to 9.3)	9.3 ± 0.2[c] (6.9 to 12.4)

Means followed by the same letters are not significantly different from one another (comparisons made using Bonferroni 95%CI).

6.4.2 Chill coma recovery and activity recovery

As the temperature increased from the chill coma temperature to the rearing or acclimation temperature, the organisms regained the ability to move their appendages and then walk. The mean temperatures at which the individuals first entered chill coma recovery and activity recovery, are shown in Table 6.1.

There were differences in the temperatures at which the parasitoid and aphid treatment groups began to recover following chill coma (χ^2=6145.05, DF=3, P<0.001). Both non-acclimated and acclimated parasitoids entered chill coma recovery at significantly lower temperatures (-5.2 and -7.3°C respectively) than both aphid treatment groups (chill coma temperatures ≥ 7.5°C) and exposure to the acclimation regime resulted in organisms regaining the ability to move at lower temperatures following chill coma. The shape and scale parameters of the distributions were also different (χ^2=27.09, DF=3, P<0.001), with acclimated aphids having the smallest range of chill coma recovery values.

Differences were observed in the temperatures at which the treatment groups regained the ability to walk following chill coma (χ^2=684.01, DF=3, P<0.001). As with chill coma recovery, parasitoids were able to regain locomotory function at lower temperatures (≤6.1°C) than aphids (≥9.3°C) and exposure to the acclimation regime significantly lowered the activity recovery temperatures in both species. The shape and scale parameters of the treatments also differed (χ^2=117.12; DF=3; P<0.001), with non-acclimated aphids having the greatest range of activity recovery values, and acclimated parasitoids the smallest.

6.4.3 CTmax and heat coma

As the temperature increased, the insects lost the ability to move their limbs in a coordinated manner and were therefore unable to walk (CT_{max}). A further increase in temperature resulted in complete immobility (i.e. heat coma).

There were differences in the temperatures at which the organisms entered CT_{max} (χ^2=94.04, DF=3, P<0.001) (Table 6.2), with non-acclimated and acclimated parasitoids retaining the ability to walk at higher temperatures (41.4 and 41.2°C, respectively) than non-acclimated and acclimated aphids (39.1 and 37.8°C, respectively). Exposure of individuals to 10°C for 7 d had no effect on the temperatures at which the parasitoids stopped walking, but resulted in a lower CT_{max} in the aphid treatments. There were no differences in the shape and scale parameters of the experimental groups (χ^2=1.90, DF=3, P=0.59).

Differences were also observed for the temperatures at which the organisms entered heat coma (χ^2=178.44, DF=3, P<0.001) (Table 6.2), with non-acclimated parasitoids having the highest heat coma temperature (44.1°C) and acclimated aphids the lowest (41.8°C). Non-

acclimated parasitoids and acclimated aphids had similar heat coma temperatures (≈43°C). There were further differences in the shape and scale parameters of the experimental types (χ^2=63.46, DF=3, P<0.001) with non-acclimated adult *L. testaceipes* having the greatest range of heat coma values.

Table 6.2: Mean (± SE) and range (in brackets) of temperatures at which non-acclimated and acclimated adult *Lysiphlebus testaceipes* and non-acclimated and acclimated second instar *Aphis fabae* lose the ability to walk (CT_{max}) and enter heat coma (n = 30 to 36 for each treatment group).

Treatment	CT_{max} (^oC)	Heat coma (^oC)
Non-acclimated adult parasitoids	41.4 ± 0.3^c (38.2 to 44.2)	44.1 ± 0.3^c (39.4 to 47.1)
Acclimated adult parasitoids	41.2 ± 0.3^c (36.5 to 43.3)	$43.4 \pm 0.1b^c$ (41.9 to 45.3)
Non-acclimated second instar aphids	39.1 ± 0.3^b (36.4 to 41.2)	43.0 ± 0.1^b (42.2 to 44.1)
Acclimated second instar aphids	37.8 ± 0.3^a (35.2 to 40.2)	41.8 ± 0.1^a (39.9 to 42.5)

Means followed by the same letters are not significantly different from one another (comparisons made using Bonferroni 95%CI).

6.4.4 Walking speed

For non-acclimated treatments, Scheirer-Ray-Hare tests showed significant effects of both treatment (H=310.4, DF=1, P<0.001) and temperature (H=326.0, DF=9, P<0.001) on the walking speeds of the experimental groups (Fig. 6.1). The same was true of the effect of treatment (H=181.3, DF=1, P<0.001) and temperature (H=218.0, DF=5, P<0.001) on acclimated organisms (Fig. 6.1). For non-acclimated treatments, parasitoids had faster walking speeds than aphids at 0 (U=1702, P<0.001) and 20°C (U=630, P<0.001) with a similar level of significance at all intervening temperatures (2.5, 5.0, 7.5, 10.0, 12.5, 15.0 and 17.5°C). For acclimated treatments, parasitoids had faster walking speeds than aphids at 0 (U=2379, P<0.001) and 10°C (U=2380, P<0.001), with a similar level of significance at 2.5, 5.0 and 7.5°C. Differences were also identified between the acclimated and acclimated

treatments, with acclimation significantly increasing the walking speeds of the parasitoids between 0 (U=2082, P<0.001) and 10°C (U=2161, P<0.001), again with a similar level of significance at 2.5, 5.0 and 7.5°C and the walking speeds of the aphids between 2.5 (U=1456, P=0.006) and 10°C (U=1870, P<0.001) with a similar level of significance at 5.0 and 7.5°C.

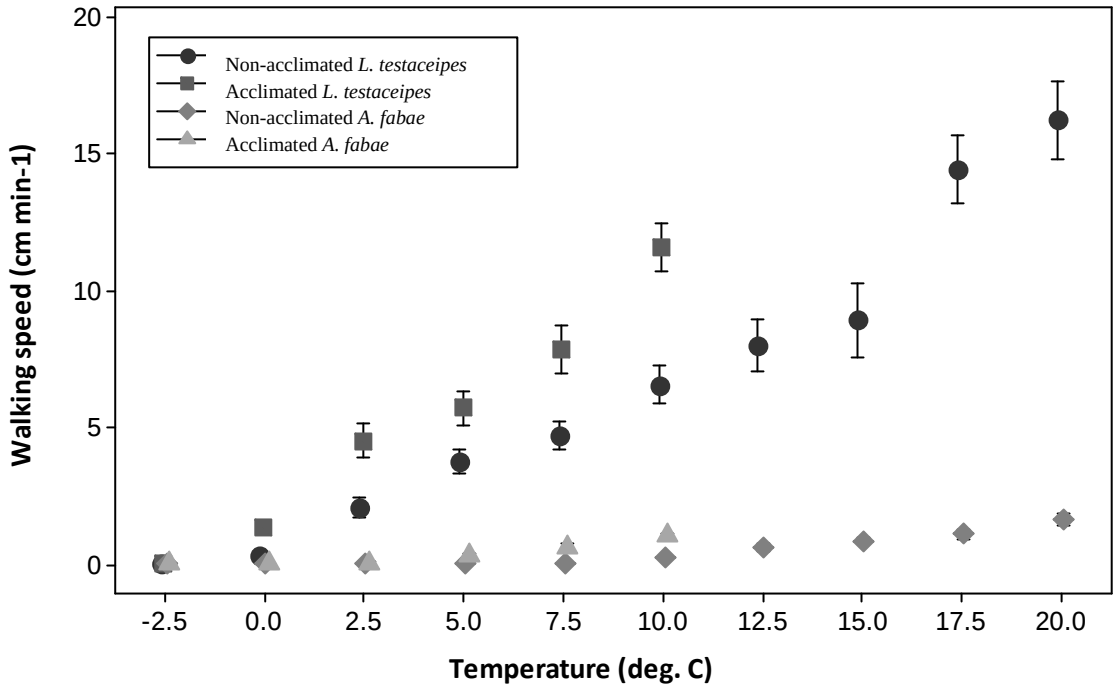


Figure 6.1: Mean walking speed (±1 SE) of non-acclimated and acclimated adult *Lysiphlebus testaceipes* and second instar *Aphis fabae* at a range of constant temperatures (n = 30 for each sample).

6.5 DISCUSSION

When identifying a potential control agent, it is important to assess the safety of the organism, in terms of its potential impact on the environment, and its efficacy against the target host or prey species. The camera setup described in this study can provide an indication of both of these factors. For example, in temperate regions, biocontrol agents that are used within glasshouses are thought to pose little or no threat to the native ecosystem, if it can be shown that climatic conditions would act as a barrier to the establishment of escaping

individuals (Tullett *et al*., 2004). Through the identification of thermal tolerance traits using the camera set-up described, an indication of cold tolerance and, therefore, establishment potential of these species can be gleaned. These species-specific characteristics can also be used to determine the ability of the predator or parasitoid to limit pest populations successfully. For example, whilst the usefulness of a predator or parasitoid can be affected by a number of factors, the control agent's activity relative to that of their prey can have a great effect on its efficacy. This chapter focuses on these characteristics through examining the thermal thresholds of *L. testaceipes* and comparing them to those of a host species, *A. fabae*.

Lysiphlebus testaceipes was included in the first compilation of the EPPO Positive List in 2001, fulfilling the criteria of having been used for 5 years or longer in at least five countries, in the EPPO region, with no reports of any negative effects. When the list of species was reviewed by the EPPO panel (reactivated in 2008 in collaboration with the IOBC-WPRS), *L. testaceipes* was removed from the list because of increasing concern about the species' expanding geographic range and the displacement of native parasitoids (Tremblay, 1984; Pike *et al*., 2000; Starý *et al*., 2004). Reasons cited for the ability of *L. testaceipes* to achieve such a dominant status included factors such as its ability to utilise a wide range of host species (Mackauer & Starý, 1967; Reitz & Trumble, 2002), greater longevity and a female-biased sex ratio (Marullo, 1987).

In this study, *L. testaceipes* was more thermally tolerant than *A. fabae* at both ends of the temperature spectrum, retaining the ability to walk at temperatures above and below those which rendered its host immobile. In addition, from 0 to 20°C, the parasitoids had faster walking speeds than *A. fabae*, suggesting that they would be able to locate their hosts quickly and efficiently. This is important because, whilst *L. testaceipes* has the capacity to fly, its host-searching technique involves the seemingly random searching of leaves, followed by aphid detection through antennal contact (Sequeira & Mackauer *et al*., 1996). Thus, the ability to move quickly across the surface of the leaf and along leaf stalks, under a range of conditions, would increase the efficacy of this search period, consequently resulting in an increased rate of parasitism.

The data obtained in this study indicate that *L. testaceipes* would constitute an effective control agent against aphid species, with similar activity profiles to *A. fabae*, in a range of environments from the cooler climes of northern Europe to the warmer regions of the

Mediterranean. Furthermore, these findings also provide some insight into the increasing habitat range of *L. testaceipes*. Whilst early reports indicated that the distribution of *L. testaceipes* might be limited by an inability to overwinter in temperate climates (e.g. Volkl, 1989), the data presented here suggest a relatively high level of cold tolerance, with parasitoids retaining locomotory function at temperatures as low as 0°C. These results are consistent with the observations of range expansion into cooler areas such as those of North America (Pike *et al*., 2000; Starý *et al*., 2004) and the northern Korean Peninsula (Starý *et al*., 2004).

To investigate further the efficacy of this candidate control agent, investigations into the ease of movement of the parasitoid across different leaf substrates could be carried out. From my personal observations, it is clear that parasitoid movement is impeded by the presence of hairs on a leaf. It, therefore, follows that the efficacy of host location on a hairy leaf substrate would be lower, thereby decreasing the rate of parasitism. The video-capture set-up described could easily be adapted to analyse the effect of different leaf substrates on efficacy by placing leaf discs on the base of the arena (Hughes *et al.*, 2010).

With regard to the establishment potential of the parasitoid, it is evident that under typical northern European winter conditions *L. testaceipes* would be able to move, allowing it to locate hosts, find shelter and avoid natural enemies, at least to some extent. In conjunction with these observations, a related study (Hughes *et al*., 2010) of the cold tolerance of *L. testaceipes* has shown that this parasitoid is able to develop and parasitize hosts in winter in such climates. For parasitoids surviving the winter, increasing temperature in spring would then allow significant dispersal of individuals from their overwintering sites, both as adult parasitoids and as eggs or larvae within alate aphids (Starý, 1988). With a host range of 100 aphid species (Mackauer & Starý, 1967, French *et al*., 2001, Tang *et al*., 2002), including some known to overwinter in the UK and other temperate regions, it seems reasonable to predict that *L. testaceipes* would be able to establish in northern Europe.

CHAPTER 7

General Discussion

7.1 INTRODUCTION

It has been estimated that arthropod pest species cause £1.98 bn (based upon exchange rate on 07/11/10) of damage to crops every year in the UK alone (Oerke *et al.*, 1994). Not surprisingly, a great deal of emphasis is placed on research into limiting this damage, thereby preventing yield and economic losses. Over the last 60 years, pest control strategies have mainly focussed on the development and use of toxic chemicals to control economically damaging organisms. Whilst pesticides remain a valuable form of pest control, concern about the impact of these substances on the environment and both consumer and producer health, coupled with the increasing cost of pesticide development and registration (van Lenteren & Manzaroli, 1999; van Lenteren & Woets, 1988), have necessitated the need for, and development of, alternative methods.

Biological control, the manipulation of naturally occurring predation, parasitism and herbivory, of one species by another, is one such alternative. This method of pest reduction pre-dates the modern pesticide era, with the first record of its use traced back to 300 AD when ancient Chinese civilisations exploited the voracious appetite of predatory ants to control pest species in citrus orchards (van Lenteren, 2005). More recently, following several well-documented successes (see Caltagirone, 1981; reviewed by Bellows & Fisher, 1999), biocontrol has become a world-wide phenomenon with more than 5000 introductions made against arthropod pests in 196 countries or islands over the last 120 years (Bale *et al*., 2008). However, in spite of the large numbers of introductions, with very few reports of impacts on non-target organisms, the last 20 years has been characterised by increasing regulation of non-native species (van Lenteren, 2006).

In the UK, since the discovery of naturalised populations of *Neoseiulus californicus* McGregor (Acari: Phytoseiidae) (Jolly, 2000) and frequent sightings in winter outside of

glasshouses of the predatory mirid *Macrolophus caliginosus* Wagner (Hemiptera: Miridae) (Hart *et al.*, 2002b), there has been a move towards a more precautionary approach to granting of licences (permits) for introduction and release of non-native agents. Thus, whilst previous risk assessments often relied on 'climate matching' as a proxy for cold hardiness, the success of a licence application is now often dependent on the submission of a comprehensive study, including direct assessments of the biology and overwintering potential of the candidate control agent.

This project focuses on two candidate control agents, *Nesidiocoris tenuis* Reuter (Hemiptera: Miridae) and *Lysiphlebus testaceipes* (Cresson) (Hymenoptera: Braconidae, Aphidiinae), primarily studying their establishment potential under northern European conditions through a series of laboratory and field trials.

7.2 *NESIDIOCORIS TENUIS*

Analysis of the laboratory and field data obtained for *N. tenuis* clearly highlighted an inability to establish in temperate regions such as the UK. Firstly, experiments demonstrated that *N. tenuis* would have restricted developmental ability in these regions, with a maximum of one generation possible in an average year and little or no development between the months of October and April. Thus, in order to survive and establish in the UK, this species would have to endure 6 months of temperatures below its developmental threshold. Also, laboratory studies failed to identify a diapause trait in this species.

Further laboratory investigations highlighted the species' lack of cold tolerance, with mirids exhibiting substantial pre-freeze mortality and showing limited survival at 5°C, with 50% mortality occurring in all treatment groups within 10 d. These results were then substantiated by field trials which confirmed that *N. tenuis* lacked a level of cold tolerance required for even a limited period of winter field survival, with 100% mortality occurring in all treatment groups within 4 weeks.

Further laboratory investigations into the thermal thresholds of *N. tenuis*, using a technique described by Hazell *et al.* (2008), permitted analysis of the mirid's behaviour at temperatures more likely to be experienced in the UK. It was clear from these data that the species would

have very limited dispersal abilities during spring, autumn and winter, with mirids unable to maintain locomotory function at temperatures below 3°C, or fly at temperatures below 10°C. In conjunction with the cold tolerance results, these data indicate that any effect that *N. tenuis* might have on the ecosystem would, at most, only be transient and localised. Therefore, in terms of the data requirements for the commercial release of non-native species in the UK and other EU countries with a similar climate, *N. tenuis* is likely to constitute a 'safe' biological control agent in such regions.

7.3 *LYSIPHLEBUS TESTACEIPES*

Investigations into the establishment potential of *L. testaceipes* highlighted considerable cold tolerance, with $LTemp_{50}$ values indicating an ability to survive exposure to temperatures significantly lower than those routinely experienced throughout winter in northern Europe. In addition to this, with a relatively low developmental threshold of 5.8°C, it was estimated that the parasitoid would be able to complete an average of 9.8 generations per year (calculated using temperature data from 1993-2007), with some development possible throughout the winter months. Field trials verified this assumption, with acclimated parasitoid larvae and pupae, within living and mummified aphids, continuing to develop throughout the 70 d of winter field exposure. Furthermore, adults emerging from mummies in the field were shown to be reproductively viable and capable of parasitism. Thus, it would not be necessary for any escaping individuals to survive outside glasshouses until the following spring, as the population could be maintained by their progeny. As a result of this, although *L. testaceipes* is a species with considerable cold hardiness, it would not be quantifiable by the $LTime_{50}$ at 5°C for individual life stages (Fig 5.11). Nevertheless, whilst conducting the LTime experiment (see sections 5.3.6 & 5.4.5), it became evident that the parasitoid had considerable cold tolerance as a result of its ability to develop at 5°C. It was, therefore, clear that winter field trials to assess the developmental and reproductive potential of this species were necessary. Thus, whilst the survival of a non-native candidate control agent at this temperature remains a viable means of ascertaining cold tolerance, in cases where the species continues to develop, additional investigations are necessary.

In terms of the data requirements for the commercial release of non-native species in the UK and other EU countries, it seems clear that during a milder winter temperature would not act as a barrier to the establishment of *L. testaceipes*. Thus, the survival of an outdoor population of the parasitoid in the UK is very much dependent on the availability of a suitable host species and ambient temperatures favouring movement, and thus host location, over the winter period. Consequently, further investigations were carried out to investigate this.

From the field trials it was evident that newly emerged adult parasitoids could survive for up to 5 weeks in the field under winter conditions. As investigations into the thermal thresholds of *L. testaceipes* indicated an ability to move at relatively low temperatures (CT_{min} = -0.1°C), it is likely that the parasitoids would experience favourable conditions for movement and host location during this time. With a host range of approximately 100 aphid species (Mackauer & Starý, 1967; French *et al*., 2001; Tang *et al*., 2002), including some known to overwinter in the UK and other temperate regions, it seems likely that *L. testaceipes* would be able to establish in northern Europe and would, therefore, not constitute a 'safe' biocontrol agent in temperate regions.

7.4 IMPORTANCE OF THE DIRECT ASSESSMENT OF COLD TOLERANCE

With the inclusion of the results presented in this thesis, the protocol established by Hatherly *et al.* (2005) for screening non-native biocontrol agents has now been used to identify the cold tolerance and establishment potential of 10 non-native candidate biocontrol agents, including three species of predatory mirid: *N. tenuis, Dicyphus hesperus* Knight (Hemiptera: Miridae) and *M. caliginosus*. Comparisons of the results obtained for these mirids emphasise the value of thermal data as a screen for establishment and highlight the dangers of using climate origin as a proxy for the direct assessment of cold tolerance.

Whilst the three mirid species investigated are very similar physiologically, with both *M. caliginosus* and *N. tenuis* originating from the Mediterranean region, they have markedly different abilities to tolerate the cold. This can be seen in the species' developmental thresholds and their annual voltinism under typical UK conditions (Table 7.1). Whilst *N. tenuis* has a relatively high developmental threshold and is, therefore, only capable of one

generation per year, both *D. hesperus* (Hatherly *et al*., 2008) and *M. caliginosus* (Hart *et al*., 2002b) can develop through two generations over the same time period.

Table 7.1: Developmental thresholds (DT) of three mirid species estimated by extrapolation of weighted linear regression.

Species	DT (°C)	Day degree requirement	Annual voltinism
*Dicyphus hesperus**	8.0**	518**	2
*Macrolophus caliginosus****	7.7	495	2.3 (2)
Nesidiocoris tenuis *****	12.9	278	1.4 (1)

Data from: * Hatherly *et al*. (2008); ** Gillespie and Sanchez (2004); *** Hart *et al.,* (2002b); and **** Hughes *et al.,* (2009).

In addition to this, whilst studies failed to identify a diapause trait in *M. caliginosus* or *N. tenuis*, the ability of *D. hesperus* to enter diapause means that, in the absence of sufficient day degrees for development, the organism would be able to survive the winter in its enhanced cold-hardy state.

Comparisons of $LTime_{50}$ at 5°C offered further indication that the cold tolerance of the three species differed significantly. Whilst 50% mortality occurred in all *N. tenuis* treatment groups within 9 d, 50% of *M. caliginosus* and *D. hesperus* survived for 32.4 and 100 d respectively. The relationship between survival at 5°C and field survival (Fig. 7.1) indicates that the three species of mirid would have markedly different abilities to tolerate winter field temperatures. As predicted, *N. tenuis* was shown to lack the cold tolerance required for even limited winter field survival in temperate climates (Hughes *et al.,* 2009), whilst 3% of nymphal *M. caliginosus* (Hart *et al.,* 2002) and 5 to 50% of individuals from all treatment groups of *D. hesperus* (Hatherly *et al.,* 2008) were able to survive throughout a colder than average winter (Table 7.2).

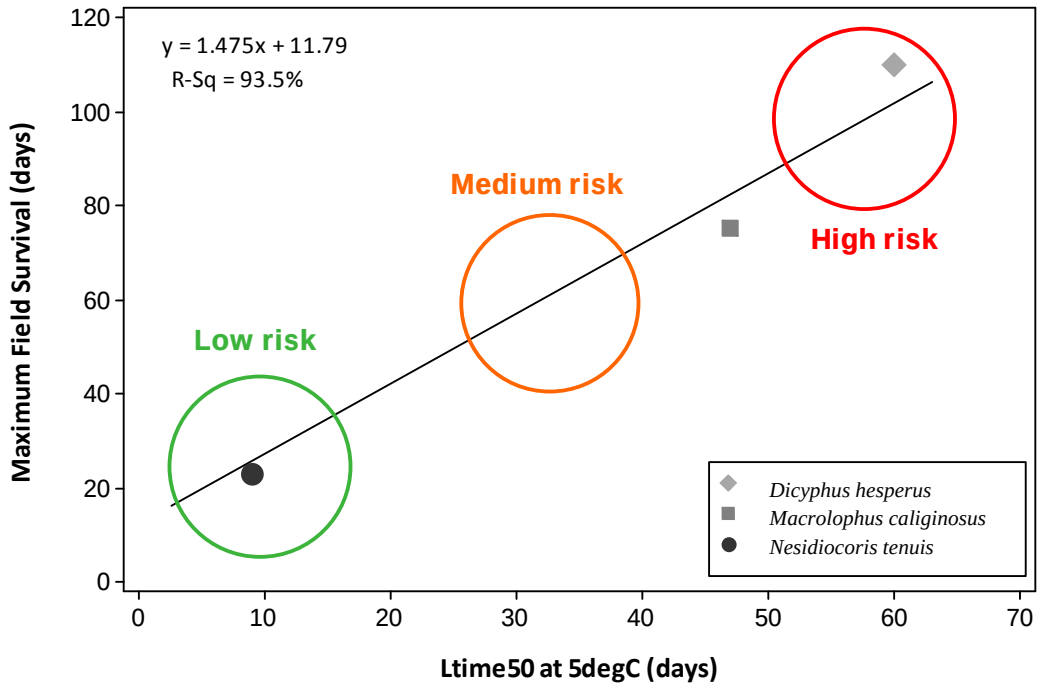


Figure 7.1: Relationship between $LTime_{50}$ at 5°C and the maximum field survival time. (Figure adapted from Figure 1.4, with some data points omitted for clarity). Sources of data: *Dicyphus hesperus* (Hatherly *et al.*, 2008), *Macrolophus caliginosus* (Hart *et al.*, 2002b), *Nesidiocoris tenuis* (Hughes *et al.*, 2009).

Table 7.2: Comparison of survival of different life stages of three non-native biocontrol agents at the end points (in brackets in d) of field experiments during UK winters.

Species	Life stage (all fed)	Survival (%) at the end of the experiment
*Dicyphus hesperus**	Non-diapausing adult	15 (148)
	Diapausing adult	50 (140)
	Nymphs	5 (140)
Macrolophus caliginosus†	Non-diapausing adult	0 (75)
	Nymphs	3 (200)
Nesidiocoris tenuis‡	Non-diapausing adult	0 (23)
	Nymphs	0 (23)

Data from: * Hatherly *et al.* (2008); † Hart *et al.* (2002b); and ‡ Hughes *et al.*, (2009).

The combination of these data placed *N. tenuis*, *M. caliginosus* and *D. hesperus* in the low, medium to high and high risk categories, respectively, (Fig. 7.1) in terms of ability to establish in temperate regions such as the UK, thereby highlighting the importance of the direct assessment of cold tolerance.

7.5 OVERALL CONCLUSION

Whilst biological control is widely acknowledged to be a green alternative to chemical pest control, it is evident that there needs to be some form of regulation to ensure the protection of native species. However, as many biological control companies are relatively small with limited funding available for research and development, it is essential that the level of regulation is proportional to the risk. The ERA described by van Lenteren and Loomans (2006) offers an efficient, flexible and cost effective means of assessing these potential risks. The flexibility within the system allows a species to be investigated in accordance with its intended use. Thus, if the species is to be used outdoors in a Mediterranean climate, where establishment and year round survival is generally guaranteed, the risk assessment would focus mainly on the host and habitat range of the organism. In situations where control agents are to be released within glasshouse environments in cool or temperate climates, the main focus would be on the cold tolerance of the individuals. In these cases, if it became clear that temperature would act as a barrier to the establishment of any escaping invertebrates, then it would not normally be necessary to investigate host or habitat range (Hughes *et al*., 2009). Therefore, for releases into temperate environments such as the UK, the ability to investigate quickly and accurately the cold tolerance and establishment potential of a candidate control agent is of utmost importance (Hatherly *et al*., 2005).

It is hoped that the work presented in this thesis will help to increase the efficacy of such investigations. The results obtained were found to support the correlation between survival of non-native invertebrates at 5°C and duration of winter field survival. It is therefore foreseeable that costly, time consuming and potentially risky (in terms of accidental release) field trials could be replaced by this relatively easy to measure laboratory index of cold tolerance ($LTime_{50}$ at 5°C) (Hatherly *et al*., 2005, 2008). Not only would the routine use of this protocol reduce the likelihood of any adverse effects of the use of non-native biocontrol

agents in the UK, but it would also increase the efficacy and cost effectiveness of the licensing procedure (Hatherly *et al*., 2005), potentially leading to an increase in the uptake of this green technology.

In addition, the procedure developed by Hazell *et al.* (2008), to identify behavioural and physiological thresholds in invertebrates, has proved to be a valuable tool in the assessment of the potential efficacy of candidate control agents within glasshouses and the potential dispersal and host-finding abilities of escaping individuals.

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