BUMBLEBEE ECOPHYSIOLOGY: ASSESSING THE IMPACTS OF CLIMATE CHANGE AND PESTICIDE USE ON *BOMBUS TERRESTRIS AUDAX* AND *B. T. DALMATINUS*

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ABSTRACT

Climate change is altering the phenology of bumblebees in parts of the UK, with worker activity recorded during winter. This presents a unique set of physiological challenges to bumblebees, as they are typically exposed to exclusively summer conditions. The aim of this thesis was to assess the capacity for winter activity and survival in the UK-native bumblebee *Bombus terrestris audax* (Harris, 1780) (Hymenoptera: Apidae) and the commercially-imported *B. t. dalmatinus* Dalla Torre, 1882 (Hymenoptera: Apidae). Cold tolerance assessments indicated that both subspecies were physiologically ill-adapted to winter temperatures. However, both species were found to undergo Rapid Cold Hardening (RCH); the first evidence of RCH in Hymenoptera. Thermal activity thresholds (CTmin, chill coma and chill coma recovery) were significantly lower in *B. t. audax* than *B. t. dalmatinus*. However, only *B. t. dalmatinus* was able to lower these thresholds as a result of acclimation. This highlights the potential for competition between the subspecies. Field experiments showed a lack of winter-active bumblebees in Birmingham, and an inability of commercial colonies to survive winter. Finally, this thesis presents the first evidence to suggest that sub-lethal neonicotinoid exposures impair bumblebee activity at low temperatures. Results are discussed in the light of climate change, pesticide use and the bumblebee pollinators in future climate scenarios.

“Every kid has a bug period... I never grew out of mine.”

E. O. Wilson.

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CHAPTER 1
INTRODUCTION

1.1. INSECT–ENVIRONMENT INTERACTIONS IN A CHANGING CLIMATE

Anthropogenic climate change is altering the Earth's biota (Parmesan, 2006) causing range shifts, extinctions and changes to the phenology of organisms. In a meta-analysis investigating the timing of events such as first flowering, tree budburst and the arrival of migrant birds and butterflies, Parmesan and Yohe (2003) found spring events were advancing by an average of 2.3 days per decade. The same study also found an average northwards shift of 6.1km per decade in birds, butterflies and alpine herbs. Climate warming has also led to an increase in summer plant growth, winter respiration and an extended growing season, particularly in Northern Europe (Myneni *et al*., 1997). With a predicted increase in mean global surface temperature of between 0.3 and 0.7°C (for the period 2016 to 2035), these changes are expected to continue (IPCC, 2014).

Insects are predicted to be most affected by climate change given their dependence on ambient temperature and the requirement that life histories are synchronous with environmental conditions, especially in seasonal climates. Both spatial and temporal range shifts have been observed as a result of climate change. For example, British butterflies are advancing northwards in response to climate change (Hickling *et al*., 2005), and are able to

emerge earlier in spring, with the orange tip butterfly *Anthocharis cardamines* (L.) (Lepidoptera: Pieridae) and the red admiral butterfly *Vanessa atalanta* (L.) (Lepidoptera: Nymphalidae) emerging 17.5 and 36.3 days earlier respectively (Roy and Sparks, 2000). Svobodová *et al*. (2014) used climactic data and insect developmental thresholds to generate a CLIMEX (CLImate inDEX) model, which was used to predict pest distributions in 2055. The model predicted a 10.8 and 8.8° northwards shift in the European grapevine moth *Lobesia botrana* (Den. and Schiff., 1775) (Lepidoptera: Tortricidae) and the Colorado potato beetle *Leptinotarsa decemlineata* (Say, 1824) (Coleoptera: Chrysomelidae) respectively. Shifts of this kind may expose an increased number of agricultural crops to destruction by insect pests. For example, a northwards shift of 10.8° of the grapevine pest *L. botrana* is estimated to expose a further 4.49 million hectares of agricultural land to damage by the pest (Svobodová *et al*., 2014).

The loss of synchronicity between predator and prey, plant and herbivore, parasite and host or plant and pollinator (Parmesan, 2006) also has the potential to disrupt whole ecosystems and biological processes. This is exemplified in the winter moth *Operophtera brumata* (L.) (Lepidoptera: Geometridae) and the Sitka spruce *Picea sitchensis* (Bong.) (Pinales: Pinaceae) in the Scottish uplands. Here, moth emergence has historically been synchronised with spruce bud burst, promoting optimal larval feeding and development (Watt and McFarlane, 1991). However, a climate change induced loss of synchronicity has resulted in moths emerging up to 4 weeks ahead of spruce budburst, resulting in poor larval nutrition (Watt and McFarlane, 1991). Mismatches are predicted to be widespread, given that 59% of species are estimated to

have changed their spatial or temporal distributions in the past 20 to 40 years, (Parmesan and Yohe, 2003).

Understanding the effect of climate change on crop pollinators is important to the continued provision of pollination services (Potts *et al*., 2010a). Insect pollination is a vital ecosystem service (Kremen *et al*., 2004), responsible for improving the quality and yield of fruit crops such as apples *Malus domestica* Borkh. (Rosales, Rosaceae) and tomatoes *Lycopersicon esculentum* L. (Solanales: Solanaceae) (Velthuis and van Doorn, 2006; Garratt *et al*., 2014). It can also promote cross-pollination in self-incompatible plants, which cannot be achieved by wind alone (Klein *et al*., 2007). Memmott *et al*. (2007) modelled the interactions between 1420 pollinator species and 429 plant species after a predicted doubling of atmospheric CO_2. The model predicted a reduction in the amount of available forage across all pollinator groups, to a level of between 17–50%. It also predicted a reduction in the dietary breadth of pollinators due to a loss of synchronicity with plants (Memmott *et al*., 2007). The authors suggested this could lead to an extinction of large numbers of pollinators, plants and their beneficial interactions.

As climate change is responsible for bringing more extreme weather events (Rosenzweig *et al*., 2001; Williams *et al*., 2015), surviving pollinators are at increased risk of exposure to extremes of cold, heat, drought and precipitation with an unknown effect on their survival. Significant pollinator losses may result in poorer crop quality, a reduction in yield and a loss of economic value (Gallai *et al*., 2009).

As with many other insects, pollinators are spatially and temporally adjusting their ranges in response to climate change. Stelzer *et al*. (2010) recorded the presence of winter active bumblebees in the south of the UK, suggesting an avoidance of winter dormancy by queens and the initiation of new colonies in autumn. In order for winter-active colonies to survive, bumblebee workers must collect sufficient quantities of pollen and nectar (Stelzer *et al*., 2010). This may be possible in urban areas, where parks and gardens contain an abundance of non-native, winter flowering plants (Stelzer *et al*., 2010), and the flowering times of native plants may have altered. In a study by Roetzer *et al*. (2000) of the urban heat island effect on the flowering of snowdrop *Galanthus nivalis* L. (Asparagales: Amaryllidaceae), forsythia *Forsythia* sp. Vahl (Lamiales: Oleaceae), sweet cherry *Prunus avium* L. (Rosales: Rosaceae) and apple *Malus domestica* Mill. (Rosales: Rosaceae) in central European cities, it was found that flowering in almost all cities was significantly earlier than rural counterparts. This is in agreement with White *et al*. (2002) who found an advancement of the growing season of 7.6 days associated with urban heat island in deciduous forests of the eastern United States. From these examples, insects associated with urban areas may have access to essential forage which is unavailable to rural populations.

A further environmental challenge facing many pollinators is increased pesticide use, which has been identified as a key driver of global pollinator decline (Potts *et al*., 2010a). As most pesticide toxicity tests involve testing for acute lethal toxicity in honeybees *Apis mellifera* L. (Hymenoptera: Apidae) (Blacquière *et al*., 2012), toxicity to other pollinating species, in addition to sub-lethal effects, is often overlooked. This thesis explores the lethal and sub-

lethal effects of a pesticide on a key bumblebee pollinator (*Bombus terrestris*; Chapter 6), in conjunction with the effects of other stressors, such as low temperature.

In order to understand the impact of climate change on key pollinator species, such as *Bombus terrestris audax* and *B. t. dalmatinus*, it is important that we examine the physiological limits of thermal tolerance. In addition to mortality studies, the sub-lethal effects, such as the impacts on foraging and activity, provide ecologically relevant information about the thermal limits of the species. The following sections aim to provide an introduction to the theory and measurement of cold tolerance and activity thresholds, whilst giving a review of *Bombus terrestris* subspecies and their commercial importance and usage.

1.2. INSECT COLD TOLERANCE

Cold, in the context of insect physiology, can be defined, not in empirical temperature measurements, but as a relative term, dependent upon the species, life stage and acclimation conditions of the insect (Lee, 1989; Izumi *et al*., 2009). It can generally be described as a temperature at which the normal development of an insect is inhibited (Salt, 1961) and performance is therefore impaired (Travisano, 2000). In order for an insect to grow and develop, a specific thermal budget is required (Bale *et al*., 2002, Bale and Hayward, 2010, Hughes *et al*., 2011), and most insects are entirely dependent on passive environmental heat (poikilothermy) to fulfil their thermal budget. Thus, every aspect of their life history is dependent upon environmental temperature (Speight *et al*., 2008; Everatt *et al*., 2012).

Sub-optimal temperatures are, for at least part of the year, unavoidable for many temperate insects (Renault *et al*., 2002). Strategies to either avoid cold stress or mitigate its affects are employed by a wide variety of insects, and are essential to their survival. Several insects possess behavioural adaptations such as migration to avoid exposure to sub-optimal conditions completely, the most notable of which is the monarch butterfly *Danaus plexippus* (Linnaeus, 1758) (Lepidoptera: Nymphalidae) (Zipkin *et al*., 2012). This species undergoes an annual mass migration from the eastern and western summer populations in North America, and travels south to the coast of California (Dingle *et al*., 2005) and the east of Mexico (Brower 1986) respectively. Another example is the migration of the green darner dragonfly *Anax junius* (Drury, 1770) (Odonata: Aeshnidae) (Zipkin *et al*., 2012), which relocates from north eastern America (Freeland *et al*., 2003) as far south as central America (Paulson, 1984; Boomsma and Dunkle, 1996). These migrations occur over several generations and are therefore distinct from the return journeys displayed in many vertebrates (Zipkin *et al*., 2012).

Smaller scale behavioural responses are also employed by a variety of insects, for example, the selection of a microhabitat that buffers against low winter temperatures (Soare *et al*., 2010). For army ants *Eciton burchellii* Westwood, 1842 (Hymenoptera: Formicidae), underground habitats provide an essential means of protecting colonies from extreme temperatures and provide favourable ambient temperatures for the development of brood (Soare *et al*., 2010). This is also true for the bumblebee *Bombus terrestris*, which initiates its colonies underground, often in disused rodent holes (Goulson *et al*., 2001; Goulson, 2010). Insects lacking such behavioural adaptations have a range of physiological strategies to

minimise the effects of cold stress. These include overwintering dormancy (diapause), which will be discussed later. Here I focus specifically on cold tolerance. In this regard, insects have historically been classified into two distinct groups of either: freeze tolerance or freeze avoidance (Zachariassen 1985; Rickards *et al*., 1987; Lee 1989; Storey and Storey, 1989). Fundamental to these classifications is the ability of organisms to tolerate freezing, with freeze tolerant insects surviving below their supercooling points and freeze avoiding species suffering mortality upon freezing.

Supercooling is the ability of water or an aqueous solution to remain in a liquid state at temperatures below the environmental freezing point (Renault *et al*., 2002; Everatt *et al*., 2014). Heterogeneous ice formation and spontaneous freezing occur at the limit of supercooling, termed the supercooling point (SCP) (Wilson *et al*., 2003). At this point, exothermic latent heat of ice crystallisation is released which can be detected experimentally (Figure 1.1 and Sinclair and Chown, 2005).

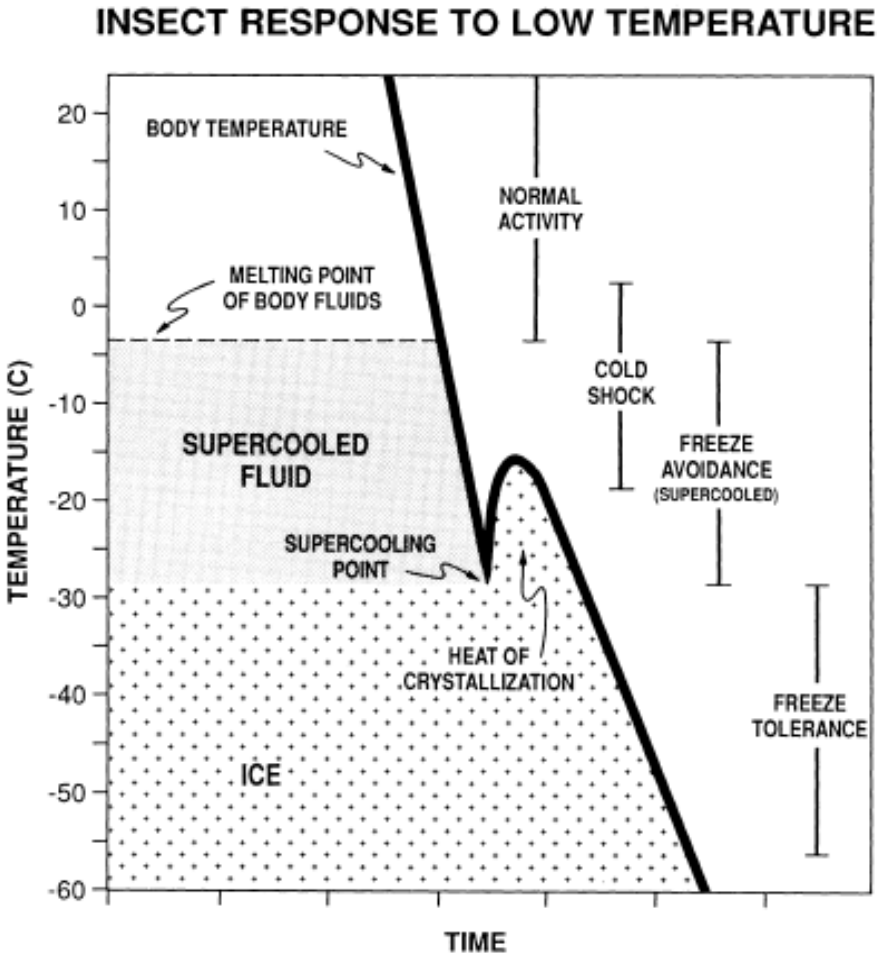


Figure 1.1 Figure and legend taken from Lee (1989). Responses of insects cooled to sub-zero temperatures. Insect body temperatures (heavy line) in relation to the melting point, the supercooling point, and the nucleation of ice in body fluids. Although the bars on the right convey general ranges of insect response to low temperatures, the top of the bar for the range of freeze tolerance and the bottom of the bar for freeze avoidance correspond to the supercooling point value illustrated in the centre of the Figure. However, a number of freeze-tolerant insects have supercooling points in the range of -8ºC to -10ºC, whereas some freeze-susceptible species supercool extensively, to -60ºC or below.

Supercooling point can be influenced by a wide variety of factors, including feeding status, diapause, life stage and metamorphosis state (Renault *et al*., 2002). Additionally, sex, thermal history, and the presence of both ice nucleating agents (which increase the SCP) and cold protective metabolites, such as sodium chloride, urea, glycerol and glucose have an effect

(Figure 1.2 and Wilson *et al.*, 2003). Kelty and Lee (1999) found when experimentally determining the SCP, the rate of cooling had a significant impact upon the result. This highlighted a need for ecologically relevant, slower, cooling rates such as 0.1 and 0.05°Cmin^{-1}.

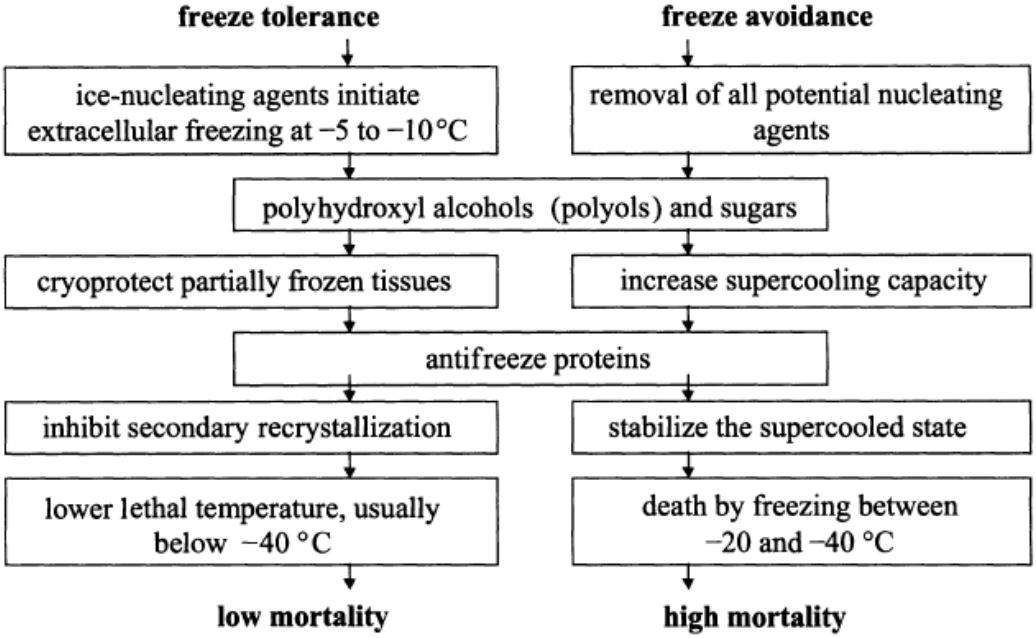


Figure 1.2 Figure and legend taken from Bale *et al.* (2002). Schematic representation of the main biochemical components involved in the freeze-tolerance and freeze-avoidance strategies of insect overwintering.

As pre-freeze mortality occurs in many insects (Bale, 1991), SCPs are not considered a reliable indicator of cold tolerance. Consequently, Bale (1993) outlined five new categories, encompassing the range of insect responses to cold: freeze tolerance, freeze avoidance, chill tolerance, chill susceptibility and opportunistic survival. These categories were based on an insect's physiological limits and this could be measured experimentally.

Besides freezing, chilling presents a potentially lethal challenge to insects (Bale and Hayward, 2010) and can be the result of acute or chronic exposure to low temperatures (Czajka and Lee, 1990; Everatt *et al*., 2014).

Freeze tolerance

Freeze tolerant insects encourage the formation of extracellular ice crystals at moderate sub-zero temperatures (typically -2 to -10°C) via the accumulation of ice nucleating agents (Bale 1996). These ice nucleating agents draw intracellular water molecules out into the extracellular medium, and form a nucleus around which they aggregate and freeze (Figure 1.3, Bale, 2002). This is a protective mechanism (Block *et al*., 1990) which allows extreme low temperature tolerance, with a freeze tolerant insect's lower lethal temperature between -40 and -80˚C (Bale 1996). Additionally, the accumulation of antifreeze proteins is used to stabilise the supercooled state (Duman *et al*., 1982) and cryoprotective polyols may lower the SCP before the freezing event as well as discouraging recurrent freezing.

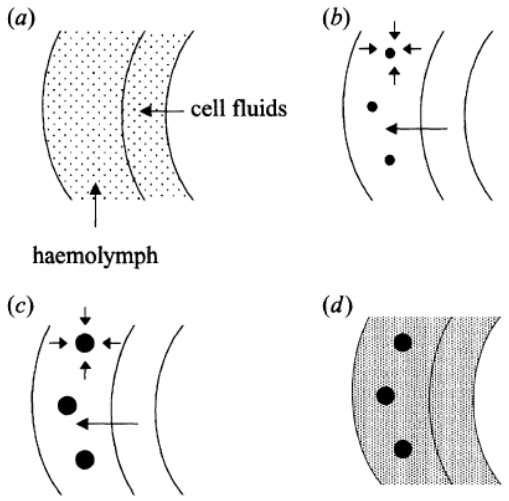


Figure 1.3 Figure and legend taken from Bale (2002). Schematic representation of the function of extracellular ice nucleating agents in a freeze-tolerant insect. Black dots indicate sites of nucleation in extracellular areas and increasing ice masses at progressively lower sub-zero temperatures. a) Equilibrium between the haemolymph and the cell fluids. b) Accumulation of INAs in the haemolymph, causing water to move out of the cell. c) Freezing occurs at sites of nucleation, and ice masses increase in size. d) All water is removed from the cell, preventing intracellular freezing.

Sinclair (1999) has further divided freeze tolerance into 4 sub-categories (partial, moderate and strong freezing tolerance, and freeze tolerance with a low SCP) depending on mortality and proximity to the supercooling point. Insects exhibiting partial freeze tolerance will suffer mortality if ice formation occurs at, or above, their SCP; moderate freeze tolerant insects will die less than 10˚C below their SCP; and strongly freeze tolerant insects can survive extreme low temperatures, with a SCP of above -15˚C and a lower lethal temperature below -20˚C. Freeze tolerant insects with a low SCP are able to survive extremely low temperatures, just below their SCP, which may be as low as -55˚C as in the beetle *Pytho deplanatus* (Coleoptera: Pythidae) (Ring 1982; Sinclair, 1999).

Freeze avoidance

Freeze avoiding insects are defined by their inability to survive intracellular and extracellular ice formation and experience mortality when their supercooling point is reached. Freeze avoidance is achieved by a lowering of the supercooling point with use of polyols and sugars, the synthesis of antifreeze proteins and the exclusion of ice nucleating agents (Bale, 1996). This exclusion removes substances such as bacteria, dust and food particles present in the gut, in addition to carbohydrates and proteins (Duman and Patterson, 1978) which may facilitate the formation of ice crystals (Zachariassen, 1982). Bale (1996) also reports that this group has little mortality above its SCP; therefore SCP is a robust measure of cold tolerance in this case, with supercooling points ranging from -20 and -40˚C. The grasshopper *Chorthippus fallax* (Zubovsky) (Orthoptera: Acrididae) is an example of a true freeze avoiding insect, as the SCP and lower lethal temperature of eggs were very similar (Hao and Kang, 2004). This situation is rare as most insect exhibit some form of pre-freeze mortality, outlined below.

Chill tolerance

Chill tolerant insects are able to survive sub-zero temperatures to a range of extents, but all experience mortality just above their supercooling point. The largest of all cold tolerance groups, chill tolerant species, can be further divided into two categories. The first encompasses highly chill tolerant organisms, such as the Antarctic mite *Halozetes belgicae* (Michael, 1903) (Sarcoptiformes: Ameronothridae), from the maritime Antarctic (Hawes *et al*., 2007). The second includes moderately chill tolerant organisms, such as the blowfly

Calliphora vicina Robineau-Desvoidy, 1830 (Diptera: Calliphoridae), from temperate climates (Saunders and Hayward, 1998).

Chill susceptibility

Chill susceptibility is often exhibited in species which fluctuate in abundance between years; their numbers under influence of the harshness of the winter and their ability to survive the cold (Bale, 1996). In the case of the adult anholocyclic aphid clones of *Myzus persicae* (Sulzer, 1776) (Hemiptera: Aphididae), *Sitobion avenae* (Fabricius, 1775) (Hemiptera: Aphididae) and *Rhopalosiphum padi* (Linnaeus, 1758) (Hemiptera: Aphididae), although they can supercool to -24˚C, a brief exposure to a moderate sub-zero temperature (between 0 and -5˚C) can cause rapid mortality (Bale, 1996).

Opportunistic survival

Insects exhibiting opportunistic survival are unable to tolerate temperatures below their developmental thresholds, and survive only by behavioural adaptations, leading them to exploit favourable microhabitats. Bale (1996) includes the housefly *Musca domestica* Linnaeus, 1758 (Diptera: Muscidae) in this grouping, as 4 days at 0°C was sufficient to cause 90% mortality in pupae (Coulson and Bale, 1990). Other examples of insects exhibiting opportunistic survival are the leafminer crop pest *Liriomyza sativae* Blanchard (Diptera: Agromyzidae), with 2 weeks at 10°C resulting in 100% mortality of pupae (Zhao and Kang, 2000), and migratory species such as the monarch butterfly *Danaus plexippus* (Zipkin *et al*.,

2012) and the green darner dragonfly *Anax junius* (Wikelski *et al*., 2006). Insects in this category may possess the ability to supercool to low temperatures, however mortality occurs at temperatures required for normal metabolism. Therefore supercooling point is again unrelated to cold tolerance. The molecular mechanisms associated with freeze tolerance, avoidance and chilling strategies are outlined in (Figure 1.4).

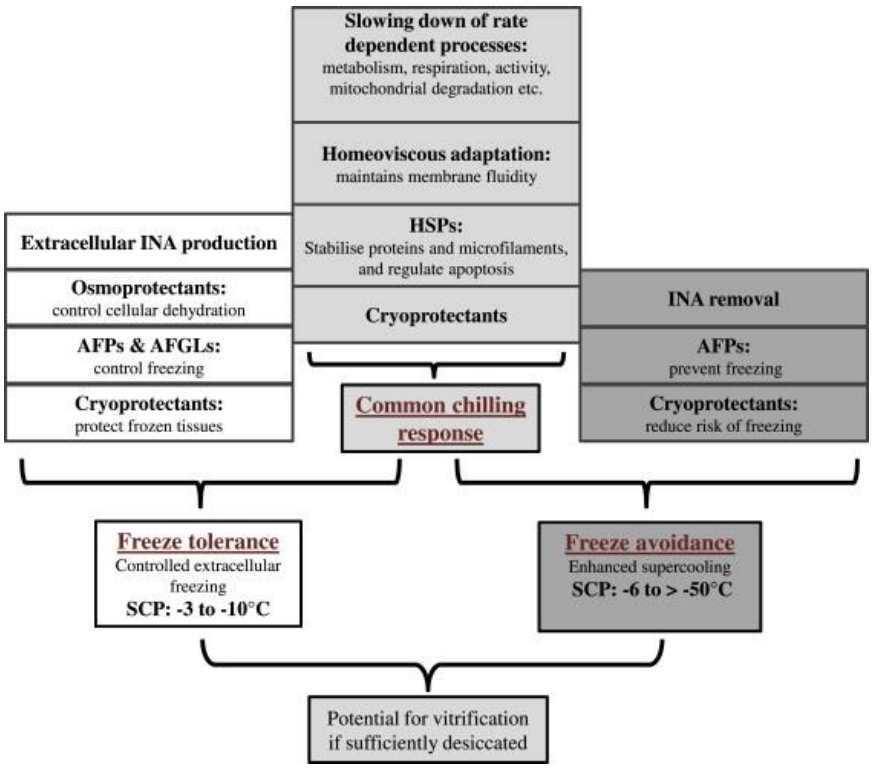


Figure 1.4 Figure and legend taken from Everatt *et al*. (2014). Schematic representation of the molecular and physiological processes underpinning chilling, Freeze Tolerance (FT) and Freeze Avoidance (FA) strategies in terrestrial invertebrates. Chilling/cold stress responses can be induced in parallel or more sequentially – the order in which temperature induces each mechanism will be species specific and potentially vary depending on the rate of temperature change. Common chilling responses (light grey) are shared by both FT and FA strategies to limit chilling injury. Fundamental differences between FT (white) and FA (dark grey) include the synthesis of ice nucleating agents (INAs) in FT vs INA removal in FA. FT insects also uniquely produce osmoprotectants to control cellular dehydration during extracellular freezing. Both strategies employ cryoprotectants (*e.g*., glycerol and trehalose) and antifreeze proteins (AFPs); and can potentially undergo vitrification. For FT species these strategies facilitate controlled freezing and limiting freezing damage, while in FA species these adaptations enhance the supercooled state/reduce the risk of ice-crystal formation. See main text for details of relevant studies.

1.3. EXPERIMENTAL MEASURES OF COLD TOLERANCE

Lethal effects of cold: lethal temperatures and lethal times

Lethal temperature can be defined as the temperature at which a proportion of a population suffers mortality as a result of a stressful experimental treatment. For example, lower lethal temperatures can be determined experimentally by exposing subjects to progressively lower temperatures, spanning between 0 and 100% mortality, rewarming and assessing mortality after a given time period. Probit analysis (Finney, 1971) is then undertaken to give an estimate of experimental temperatures resulting in 10, 50 and 90% mortality. Lethal times are much the same, although subjects are exposed to a set temperature for progressively longer times, again spanning between 0 and 100% mortality. Probit analysis then gives an estimate of experimental durations at set temperatures which result in 10, 50 and 90% mortality.

Sub- lethal effects of cold: activity thresholds

Activity thresholds provide an insight into the physiological limits of movement under different thermal conditions (Hazell and Bale, 2011; MacMillan and Sinclair 2011). Low temperature activity thresholds are measured by observing insect behaviour while temperature is decreased at a constant rate (Hazell and Bale, 2011 and Figure 1.5).

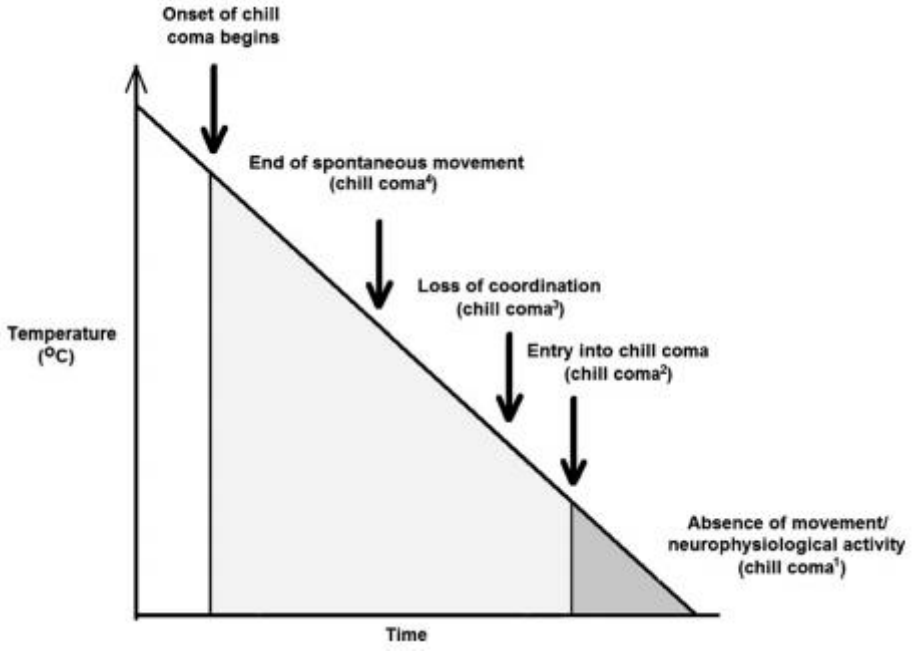


Figure 1.5 Figure and legend taken from Hazell and Bale (2011). Diagram illustrating the sequence of behavioural and physiological thresholds commonly measured in studies of chill coma. The optimum temperature range for activity is unshaded. Onset of chill coma is highlighted in light grey, beginning at the temperature at which locomotor efficiency is first compromised and ending at the temperature at which chill coma is entered (chill coma2). The threshold temperatures at which spontaneous movements end (chill coma4), coordination is lost (chill coma3), and the final burst of uncoordinated movements signal entry into chill coma (chill coma2) are highlighted. Chill coma the physiological state (chill coma1) is highlighted in dark grey.

Activity thresholds provide an ecologically relevant insight into activities which require mobility, such as foraging, mating and predator avoidance (Everatt *et al*., 2013). The experimental measures of low temperature activity thresholds are as follows:

- Critical thermal minima (CTmin). This is the temperature at which coordinated, ambulatory movement is lost (Figure 1.5; chill coma3).
- Chill coma. This is the temperature at which the ability to move an appendage is lost (Figure 1.5; chill coma1).

Low temperature thresholds are usually reversible, if freezing does not occur (Hazell and Bale, 2011). Therefore, when gradually increasing the temperature of insects in chill coma, recovery temperatures can be recorded. Experimental measures of this recovery are as follows:

- Chill coma recovery. After a period of chill coma, this is the temperature at which an insect is first able to move an appendage.
- Activity recovery. After a period of chill coma, this is the temperature at which an insect is able to regain coordinated movement.

The onset of CTmin and chill coma are the result of a disruption to neuromuscular function, resulting from an inability to maintain ion homeostasis (MacMillan and Sinclair 2011). As temperature is decreased, nerves lose the ability to generate action potentials (needed to initiate muscle contractions) and the membrane resting potentials of muscles are disrupted (MacMillan and Sinclair 2011). When examining action potentials in the locust *Locusta migratoria* (Linnaeus, 1758) (Orthoptera: Acrididae), decreasing low temperatures led to a build-up of extracellular [K^+] and a rapid depolarisation and silencing of nerve transmission (Rodgers *et al*., 2010; MacMillan and Sinclair 2011). Muscle potentials were investigated in *Apis mellifera* and *Drosophila melanogaster* Meigen, 1830 (Diptera: Drosophilidae), and a decreasing temperature was found to result in an influx of Ca^+, resulting in a disruption to membrane resting potentials and a lack of muscle contraction (Hosler *et al*., 2000; MacMillan and Sinclair 2011). The build-up of K^+ and Ca^+ in the above examples was a result of disruption to ion-motive ATPases, namely Na^+/K^+-ATPase and Ca^{2+}ATPase respectively,

which are essential in the maintenance of ion balance across membranes (Hazel, 1995; MacMillan and Sinclair 2011). The mechanisms behind chill coma recovery are poorly understood, however, recovery from chill coma is thought to be a factor in the amount of chilling injury, and the duration and magnitude of low temperatures experienced by the insect (MacMillan and Sinclair 2011). In studies where heat activity is investigated, the loss of coordinated movement is termed CTmax and the lack of appendage movement is termed heat coma (Hazell and Bale, 2011). Insects usually do not recover from heat coma, as they would do for chill coma (Piyaphongkul *et al*., 2012).

The response of insects to low temperatures depends on many factors, including feeding status, life stage, gender, the presence of ice nucleating agents and the thermal history of the organism (Renault *et al*., 2002; Wilson *et al*., 2003). Short and long term acclimation, for example, may mitigate the negative effects of low temperatures, and provide the insect sufficient time to allocate resources to minimising cold injury (Lee *et al*., 1987). The following sections outline the effects of short and long term acclimation on insects, and the resulting impact on survival.

1.4. EFFECTS OF ACCLIMATION ON COLD TOLERANCE

Short term acclimation: rapid cold hardening

The process of rapid cold hardening, first reported by Lee *et al*. (1987) and Chen *et al*. (1987), was identified when individuals of the non-diapausing, freeze intolerant flesh fly *Sarcophaga crassipalpis* Macquart, 1839 (Diptera: Sarcophagidae), elm leaf beetle *Xanthogaleruca luteola* (Müller, 1766) (Coleoptera: Chrysomelidae) and milkweed bug *Oncopeltus fasciatus* (Dallas, 1852) (Hemiptera: Lygaeidae), all experienced increased survival at low sub-zero temperatures after a short period (1 hour) of acclimation at 0°C. To investigate rapid cold hardening experimentally, a series of cold exposure experiments determine the temperature at which 80 to 90% of a subject population die, termed the 'discriminating temperature'. Fresh samples of insects are then exposed to the discriminating temperature, both with and without a prior period of chilling, for example 1h at 0°C. Differential survival is then assessed to determine the impact of a chilling pre-treatment on survival. RCH has now been discovered in a wide variety of insects, encompassing 8 orders and 26 families (Denlinger and Lee, 2010), but is yet to be identified in Hymenoptera (see Chapter 2). RCH enables insects to respond to cold stress in a matter of minutes to hours (Lee *et al*., 1987). This has adaptive significance in insects regularly exposed to sub-optimal temperatures in early spring or autumn (Czajka and Lee, 1990). The importance of cooling at ecologically relevant rates is again highlighted, as Kelty and Lee (1999) report a higher incidence of survival using cooling rates of 0.1 or 0.05°Cmin^{-1} as opposed 1.0 or 0.5°Cmin^{-1}.

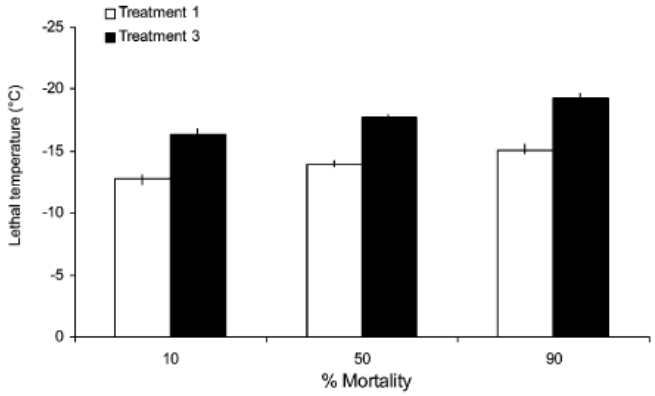
Mechanisms underpinning RCH centre on the accumulation of cryoprotectants, the stabilisation of membranes and the synthesis of heat shock and antifreeze proteins. RCH has been found to increase the production of cryoprotectants such as glucose and (Overgaard *et al*., 2007) and glycolysis metabolites such as pyruvate, glucose, alanine and glycerol (Michaud and Denlinger, 2007). Heat shock proteins (HSPs) have also been implicated in the RCH response given their ability to act as molecular chaperones, stabilise proteins and reduce aggregation (Kelty and Lee, 2001). Antifreeze proteins may also be responsible for increased survival as a result of RCH. For example, Zachariassen and s (1982) found the supercooled state was stabilised by proteins which inhibited the growth of the ice lattice, and Lee (1989) suggested that they lowered both the freezing and supercooling points. Finally, evidence suggests that RCH also plays a role in minimising cell death (Yi *et al*., 2007), mitigating the effects of cold-induced cell death.

Long-term acclimation

Acclimation is the adaptation of an organism to a single, novel, abiotic factor such as temperature (Lagerspetz, 2006), often in a laboratory setting (Everatt *et al*., 2014). This is distinct from acclimatisation, which is the adaptation to multiple, changing environmental factors, usually in the wild (Lagerspetz, 2006; Everatt *et al*., 2014). In a laboratory setting, acclimation is usually achieved by exposing an organism to a moderately low temperature (for example, 1 week at 10°C; Hatherly *et al*., 2005), which confers a decrease in cold injury and death at lower temperatures (Hart *et al*., 2002). For example, a predatory mite, *Amblyseius californicus* McGregor (Acari: Phytoseiidae), was acclimated for 1 week at 10°C, which

resulted in decreased lethal temperatures (Hart *et al*., 2002; Figure 1.6.a). The same, significant trend was found when investigating lethal times: mites were able to survive longer durations at stressful low temperatures as a result of acclimation (Hart *et al*., 2002, Figure 1.6.b).

a)



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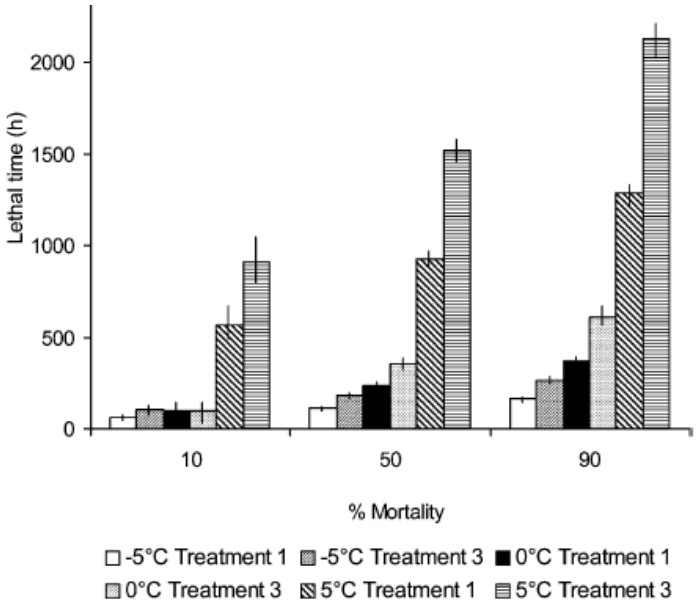

Figure 1.6 Figure and legend taken from Hart *et al*., 2002. a) $LTemp_{10,\ 50}$ and $_{90}$ (± 95% fiducial limits) of adult female *A. californicus* reared at 26°C, 18L:6D (Treatment 1) and at 19°C, 6L:18D followed by a period of acclimation for 7 days at 10°C, 12L:12D (Treatment 3). b) Lethal time (± 95% fiducial limits) of adult female *A. californicus* reared at 26°C, 18L:6D (treatment 1) and 19°C, 6L:18D with a period of acclimation for 7 days at 10°C, 12L:12D (Treatment 3) after exposure to -5, 0 and +5°C for increasing periods of time.

Acclimation also enhanced low temperature survival in third-instar *D. melanogaster* larvae, where individuals exposed to 15 and 6°C for a period of 2 days had a longer lethal time (by 50%), as opposed to those maintained at a constant 25°C (Koštál *et al*., 2013). This increase in cold tolerance was correlated with a change in the metabolic profile of the larvae, with increases in proline and trehalose, which are important in the maintenance of membranes (Koštál *et al*., 2013). Membranes are restructured for three key reasons (as described by Koštál *et al*., 2013):

- To prevent membrane breakage as a result of cell swelling (as a result of freezing) or cell shrinkage (as a result of dehydration).
- To prevent the phase transition of membranes, *e.g*. from a fluid state to a solid state.
- To promote the function of membrane proteins, such as ATPases, essential in the maintenance of ion balance (discussed in later sections).

Acclimation is therefore a key strategy used by insects to improve their cold tolerance and enhance survival at sub-optimal temperatures. Strategies such as acclimation are valuable in seasonal climates, where temperatures can range vastly between seasons (Koštál *et al*., 2011). However, during the coldest periods of the year, most insects from seasonal climates enter a form of dormancy, termed diapause, in order to survive.

1.5. OVERWINTERING STRATEGIES IN A SEASONAL CLIMATE

Insects inhabiting temperate regions are exposed to sub-optimal thermal conditions and fluctuating resources for part of the year (Koštál, 2006), the extent of which depends on the species, habitat and climate range (Bale *et al.*, 2002). To promote survival, most temperate insects have developed an environmentally regulated dormancy, termed diapause (Denlinger, 2002). Diapause allows insects to enter into a dynamic state of arrested development during adverse conditions, often continuing for a period of months (Denlinger, 2002). The 'decision' to enter diapause is not a direct response to unfavourable conditions, but is pre-emptive, with photoperiod, and, secondarily, thermoperiod, providing the 'cues' for entry (Denlinger, 2002). Diapause can occur at any developmental life stage, for example egg diapause in the silk moth *Bombyx mori* (Linnaeus, 1758) (Lepidoptera: Bombycidae) (Noguchi and Hayakawa, 2001), pupal diapause in the corn earworm *Helicoverpa zea* (Boddie, 1850) (Lepidoptera: Noctuidae) (Zhang and Denlinger, 2010) and adult diapause in the Colorado potato beetle *Leptinotarsa decemlineata* Say 1824 (Polyphaga: Chrysomelidae) (Yocum, 2001). Diapause can be obligatory or facultative: obligatory diapause occurs at the same life stage in every generation, whereas the entry into facultative diapause is not a certainty, and is often determined by environmental conditions (Bale and Hayward 2010). Diapausing insects can be freeze tolerant or freeze avoiding, but the vast majority studied to date demonstrate freeze avoidance strategies. The diapause programme progresses through a series of stages: photosensitive phase followed by the preparative phase, an entrance into diapause, diapause termination and post diapause quiescence (Figure 1.7; Denlinger, 2002; Koštál, 2006), as outlined below.

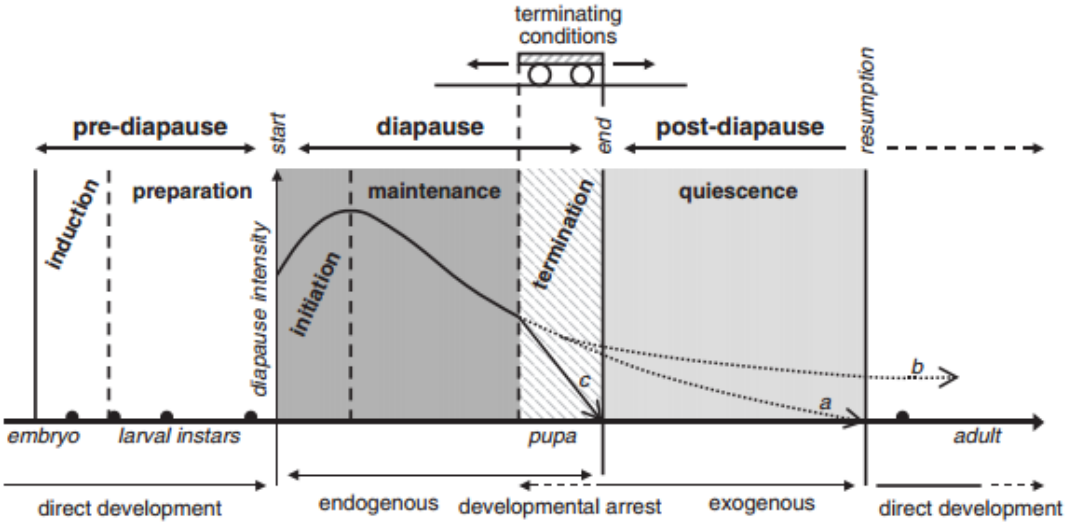

Figure 1.7 Figure and legend taken from Koštál (2006). Schematic depiction of diapause terms. Thick line with arrowhead in the lower part of the picture indicates the passage of time starting from formation of zygota to the death of one hypothetical insect individual. The points on the line delineate major ontogenetic stages (different staging must be considered in different species). Three major phases, namely pre-diapause, diapause and post-diapause, are distinguished during the diapause-including ontogeny. Further division to sub-phases, namely induction, preparation, initiation, maintenance, termination and quiescence, is indicated by vertical lines (not all the phases must necessarily be found in all species and situations). Changes in diapause intensity are schematically presented: dotted branches (a and b) apply to the constant conditions, while solid branch (c) applies to the change of environmental conditions (specific terminating conditions/stimuli coming at different physiological times).

During the photosensitive phase, pre-diapausing insects experience photoperiodic 'cues' based on the environmental light:dark ratio. The critical day length (CDL) refers to the day length which causes 50% of individuals to enter diapause (Bean *et al*., 2012). The CDL required for diapause induction is unique to each species, and even different latitudinal populations of species (Saunders and Hayward, 1998). It is detected via an endogenous 'clock' and a 'counter', able to determine the number of days an insect has experienced a specific photoperiod. The clock in *Drosophila melanogaster*, responsible for photoperiodism is governed by CYCLE/CLOCK and PER/TIM heterodimers which positively and negatively regulate the transcription of period (*per*) and timeless (*tim*) genes respectively (Ikeno *et al*.,

2010). This system is entrained to the light:dark cycle via CRYPTOCHROME. Denlinger (2002) questions the role of *per* in diapause regulation as *per* knockout mutants of *Drosophila melanogaster* can readily enter diapause but lose circadian rhythmicity (Saunders *et al*., 1989). However, recent advances have elucidated other genes which influence entry into diapause such as the period homolog *lin-42* in the nematode *Caenorhabditis elegans* (Maupas, 1900) (Rhabditida: Rhabditidae) (Tennessen *et al*., 2010) and *cyc* in the bean bug *Riptortus pedestris* (Fabricius, 1775) (Hemiptera: Alydidae) (Ikeno *et al*., 2010).

The preparative stage of diapause contains both behavioural and metabolic adaptations, designed to prepare the organism for both unfavourable environmental conditions and diapause induced metabolic changes. Adaptations include the location of suitable hibernacula, accumulation of fat reserves, synthesis of storage proteins and cuticle waterproofing (Denlinger 2002). The state of diapause can be further sub-divided into 3 groups; initiation, maintenance and termination. Diapause initiation begins with developmental arrest and metabolic suppression, which may be easily distinguished by a change of colour, moulting or the construction of a cocoon (Koŝtál, 2006) and is associated with hormonal signalling (for a review see Denlinger, 2002). Maintenance of diapause involves a continuation of metabolic suppression and, as the stage progresses, an increasing sensitivity to environmental signals which may initiate the end of diapause. However, little is known of the physiological process involved in this stage (Koŝtál, 2006).

Diapause termination occurs as a result of photoperiod, food availability, moisture or the influence of hosts or parasites before the onset of favourable conditions (Tauber and Tauber, 1976). As most individuals exit diapause within a short period, the populations are synchronised (Noguchi and Hayakawa, 2001). A state of post diapause quiescence is then entered; an environmentally responsive state in which normal development will only resume after the onset of favourable conditions. Hayward *et al*. (2005) emphasise the need for adequate thermal conditions in the termination of post diapause quiescence in *Sarcophaga crassipalpis;* pupae in the quiescent state kept at 25°C resumed development within 12 days whereas pupae maintained at 10°C remained dormant throughout the duration of the experiment (150 days).

Diapause thus plays a crucial role in the synchronisation of insect life histories with seasonal change, so that individuals, indeed populations, emerge at the appropriate times for mating and feeding (Danks, 2002; Hahn and Denlinger, 2007). In the case of pollinators, this synchronisation has added importance, as pollinators must emerge from diapause at a time when crops are ready to be pollinated (Sheffield, 2008; Miller-Rushling and Høye, 2010). As the result of an extended growing season, the leaf-cutter bee *Megachile rotundata* (Fabricius, 1787) (Hymenoptera: Megachilidae), a pollinator of lowbush blueberry *Vaccinium angustifolium* Aiton (Ericales: Ericaceae), is emerging from diapause early and developing a bivoltine trait (Sheffield, 2008). This means, however, that there are fewer individuals available at the start of their second colony cycle: the time at which crop pollination is needed the most (Sheffield, 2008). Short-term studies such as this provide useful information about

insect-plant interactions, however, they are less useful in determining trends: results can be skewed by short-term variations in environmental temperature, such as unusually cold winters (*e.g*. winter 2010 in Europe; Cattiaux *et al*., 2010). A long-term study by Hodgson *et al*. (2011) was able to predict future phenological trends by analysing data collected over a 33-year period. It analysed the transect data of 15 species of UK butterfly, collected between 1973 and 2000, to determine if the species had altered their phenologies during the period, and if their phenologies correlated with environmental variables. General additive models were then created and used to predict future phenological changes as a result of climate change. The phenological data showed changes in the distribution of 6 species, but phenological changes in 9 species. Of these 9 species, 4 of these (the small white *Pieris rapae* (L.), large white *P. brassicae* (L.), Adonis blue *Polyommatus bellargus* (Rott.) and wallbrown *Lasiommata megera* (L.)) were robust enough to predict future changes to the spatial and temporal abundance of butterfly species.

Photoperiod and temperature regulate phenological responses, however, there is a large level of variation between and within species, making predictions difficult. Each insect has a specific photoperiodic (and often thermal) cue in order for it to initiate a response (Tauber and Tauber, 1976). For example, the fruit fly *Drosophila melanogaster* relies on a combination of CDL (<14h light) and environmental temperature (<12°C) (Saunders *et al*., 1989), but these cues vary between individuals in a population. Identifying phenological changes in insects is essential to the continued provision of pollination services, especially in the case of pollinators (Hegland *et al*., 2009) and is further discussed in the following sections.

1.6. INSECT POLLINATION

Pollination, the act of transferring pollen from anther to stigma, is required for most plants to produce a seed set, and provides high yields of quality produce (Goulson, 2010). Pollination is primarily performed by bees, but other pollinators include butterflies, moths, flies, beetles and wasps (Kremen *et al*., 2007). The honeybee *Apis mellifera* is the most prolific and economically important commercially-managed pollinator (Klein *et al*., 2007); however, in recognition of numerous advantageous adaptations and improvements in rearing methods, bumblebees have been employed as commercial pollinators since 1988 (Inari *et al*., 2005). Numbers of exported colonies have gradually risen and in 2006, approximately one million colonies were being used by growers around the world (Velthuis and van Doorn, 2006). The following sections aim to highlight the use of bumblebees as commercial pollinators and provide a comprehensive account of the *Bombus terrestris* subspecies, focusing on *B. t. audax* and *B. t. dalmatinus*, which are the study species within this thesis.

1.7. BUMBLEBEES IN AGRICULTURE

Historically, honeybees were the sole focus of pollinator initiatives, due to their large colonies and ease of management (Goulson, 2010). However, due to the infestation and collapse of many North American colonies and the prevalence of the *Varroa destructor* mite (Tentcheva *et al*., 2004; van Engelsdorp *et al*., 2008), a range of other species is being increasingly used on a commercial scale. Bumblebees have been recognised as pollinator alternatives to honeybees, as they are also able to pollinate glasshouse and open air crops (Dafni *et al*.,

2010). The pollination of tomato crops, *L. esculentum*, has particular significance, with bumblebees responsible for the pollination of 40,000ha of tomato crops worth €12,000 million in 2004 alone (Velthuis and van Doorn, 2006). Other crops principally pollinated by bumblebees include alfalfa *Medicago sativa* L., clovers *Trifolium spp*. L., oilseed rape *Brassica napus* L., brown mustard *Brassica juncea* (L.), sunflower *Helianthus annuus* L. and fruits such as strawberry *Fragaria x ananassa* Duchesne, melon *Cucumis melo* L. and kiwifruit *Actinidia deliciosa* Boud (Goulson, 2010).

Key to their success is their ability to buzz pollinate (Buchmann, 1983). This process involves the contraction of a bee's indirect flight muscles to dislodge pollen from a flower, which is then collected and moved into the bee's corbiculae for transportation (Dafni *et al*., 2010). This technique is most useful in the pollination of *Solanaceae* (*e.g*. tomatoes, eggplants and peppers) (Kwon, 2008), with Winter *et al*. (2006) reporting that bumblebees were 400 times faster at harvesting pollen than honeybees. Buzz pollination is exclusive to four bee genera – *Bombus*, *Megachile*, *Xenoglos* and *Xylocopa* (Buchmann 1985).

Since the 1980s, advances in the development of artificial bumblebee (*Bombus terrestris*) colonies have resulted in their mass production and transportation into 57 countries, with 16 outside their native range (Ings, 2006). Greenhouse pollination is now occurring irrespective of season in the UK, with the first year-round tomato pollination occurring in 2005/2006 (Ings, 2006; Velthuis, 2002). This transport, however, occurred without risk assessment (Ings *et al*., 2005b), and resulted in bumblebee establishment in several non-native countries. For

example, bumblebees (*Bombus terrestris*) were introduced into Japan in 1991 for use in glasshouses and were imported at a rate of 70,000 per year until 2004 (Goka, 2010). After the escape and establishment of a wild population however, with unknown consequences to native species (Nagamitsu *et al*., 2007), the Ministry of Agriculture, Forestry and Fisheries prompted the use of the native bumblebee *Bombus ignutus* Smith, 1869 (Apoidea: Apidae) as an alternative pollinator (Mizutani and Goka, 2010). Establishment is also confirmed in Chile, Argentina, Israel, New Zealand and Tasmania (McFadyen and Lloyd, 2006).

The global exportation and extensive usage of *B. terrestris* is a result of its high efficiency and adaptability as a commercial pollinator. The following sections aim to explore the physiology and phenology of this species and discuss the potential impact of climate change on the pollination services it provides.

1.8. *BOMBUS TERRESTRIS*: LIFE CYCLE

The buff-tailed bumblebee, or large earth bumblebee (*Bombus terrestris*), is native to Eurasia and is characterised by its large size (10 - 28mm) and short tongue (4.5 - 6.5mm) (Dafni, 2010). Eusocial colonies are located underground, often in disused rodent holes and consist of up to 350 individuals (Goulson *et al*., 2001; Goulson, 2010). The life cycle of the bumblebee in Northern Europe has been extensively researched and is well-understood (Figure 1.8 and Sladen, 1912). In early spring, mated overwintered queens emerge from diapause, provide the nest with pollen and initiate colony development (Beekman and van Stratum, 2000; Goulson,

2010). Eggs are laid within a pollen lump and the queen alternates between pollen foraging and brood incubation, maintaining a brood temperature of between 30 and 32˚C (Goulson, 2010; Heinrich, 1975). Larvae continue to be fed until pupation (Ribeiro *et al*., 1999). After 4-5 weeks, depending on temperature and food provisions (Alford 1975; Goulson, 2010), the first workers emerge and tend to all further eggs (Sladen, 1912). Egg-laying continues until late summer when new diploid queens and haploid males are produced (Gadau *et al*., 2001). New queens then accumulate glycogen reserves, mate, locate suitable hibernacula and enter the 'diapause' state (Alford, 1969). Males and remaining workers within the colony die at the onset of winter.

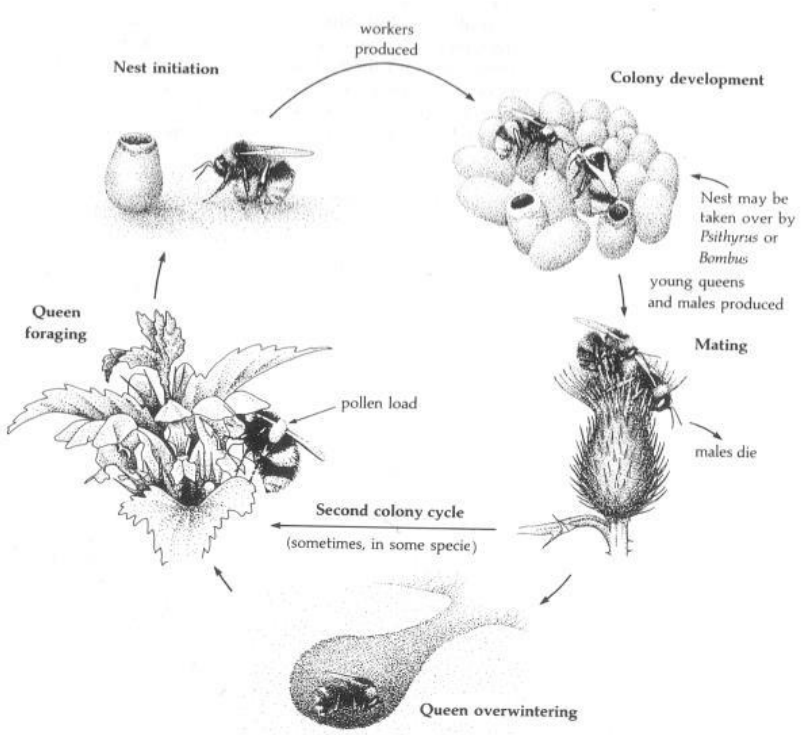

Figure 1.8 Figure and legend taken from Prys-Jones and Corbet (1991). The life cycle of *Bombus terrestris* in the UK.

Bumblebee castes consist of queens, female workers and males, with queens noticeably larger than both workers and males. This size difference can be attributed to delayed later moults in the queen's development, providing a longer feeding duration (Cnaani *et al*., 2000) and larger fat deposits (Goulson, 2010). Juvenile hormone (JH) biosynthesis is thought to be responsible for the delayed queen moult, and Cnaani *et al*. (2000) report JH synthesis rates 8 to 10 times higher in queen larvae compared to worker larvae. Caste determination occurs as early as 5 days (Cnaani *et al*., 2000). Beyond this time, larval feeding is self-directed, with larvae emitting 'hunger' signals, promoting food provision (Pereboom *et al*., 2003). Within the worker caste there is a large variability in size, with smaller workers adopting nest maintenance roles and larger workers working as foragers (Spaethe and Weidenmüller, 2002). With body size impacting thermoregulation, flight speed and nectar ingestion (Spaethe and Weidenmüller, 2002), larger bees are better adapted to the demands associated with foraging in a range of conditions. Although there exists a forager / nest worker divide, a strict age-related hierarchy (age polyetheism) as in honeybees (Seeley, 1982), is not present.

1.9. *BOMBUS TERRESTRIS*: DIAPAUSE

Queens diapause in 'hibernacula', often found on north facing slopes (Sladen, 1912), for a period of 6 to 9 months (Alford, 1969). Environmental cues are not thought to trigger diapause entry as queens inhabit dark, underground colonies where temperature, humidity and food availability are controlled by workers (Beekman *et al*., 1999). A cue has not yet been found although Beekman *et al*. (1999), suggest diapause in the species is facultative, and this is supported by the fact that non-diapausing colonies can be established. Beekman *et al*.

(1998b) found diapause survival was directly related to the pre-diapausing weight, *i.e*. the accumulated fat reserves, of the queen. Diapause has also been found to alter the characteristics of resulting colonies. For example, colonies from diapausing queens produce more workers with increased longevity, but produce fewer new queens (Beekman and van Stratum, 2000). However this result was not thought to be associated with the costs of diapause, but with the number of reproductive cycles per year (Beekman and van Stratum, 2000).

Recent reports of non-diapausing queens (Ings *et al*., 2006) and the winter activity of workers in the UK (Stelzer *et al*., 2010) add to a growing body of evidence suggesting *B. terrestris* queens can avert diapause under certain conditions, and so establish winter active colonies in the UK. This suggests queens are undergoing two reproductive cycles per year (bivoltinism), and initiating new colonies at the end of autumn. Whether this phenomenon is the result of climate warming or the hybridisation of the UK-native subspecies *B. t. audax* with commercially imported *B. t. dalmatinus* is discussed in later sections.

1.10. *BOMBUS TERRESTRIS*: THERMOREGULATION

Thermoregulation in individuals

In order to initiate and maintain flight, bumblebees must raise their thoracic temperature above 30˚C (Krogh and Zeuthen, 1941). Stone and Willmer (1989) found that *B. terrestris* queens could warm up by 1.6˚C in 10 seconds, to a stable flight temperature of 33.3 ±0.3˚C, and there is good evidence that bees can maintain their body temperature well above ambient conditions (Figure 1.9). This flight temperature is an equilibrium between heat generated and heat lost and is dependent on factors such as body conductance and ambient temperature (Goulson, 2010).

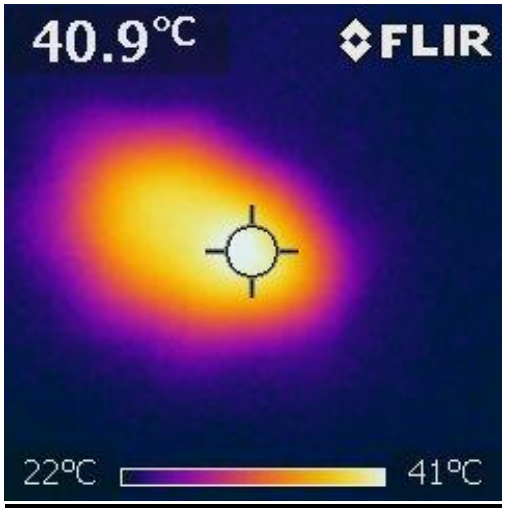

Figure 1.9 An infrared, thermal image of a *Bombus terrestris audax* worker. (Taken by E.L. Owen, using a FLIR E, compact thermal imaging camera).

Many studies have shown that, as temperature decreases, metabolic rate increases as a result of increasing heat generation. This inverse relationship between temperature and metabolic rate has been reported in the anthophorid bee *Centris pallida* Fox (Hymenoptera: Apidae)

(Roberts and Harrison, 1998) and the orchid bee *Euglossa imperialis* Cockerell (Hymenoptera: Apidae) (Borrell and Medeiros, 2004). However, the alpine bee *Andrena bicolor* Fabricius (Hymenoptera: Apidae) lacked any form of endothermy when tethered but did generate heat during free-flying foraging (Herrera, 1995). In agreement with this, Woods *et al*. (2005) reported endothermy only in free-flying bees, and not in bees requiring prompting or agitation to fly. Finally, Harrison and Fewell (2002) found low levels of heat generation in *Apis mellifera* individuals that were tethered, those that were winter acclimated and those without a foraging-incentive. These studies suggest that endothermy depends on the perceived ability of a bee to obtain floral rewards.

As heat loss occurs at a faster rate in cold and wet conditions, foraging bumblebees must invest more in poor conditions (Newsholme *et al*., 1972). Bees try to minimise heat loss to the environment and prefer to land on warmer flowers, using colour as an indicator of warmth (Dyer *et al*., 2006). This heat is generated by simultaneous contraction of flight muscles in the thorax, as opposed to alternating contraction when flying (Goulson, 2010). This generates an audible buzzing which is correlated with metabolic heat flux (Schultze-Motel and Lamprecht, 1994) and a pumping of the abdomen (Stone and Willmer, 1989). Flight muscle metabolism is thought to be generated by actinomyosin ATPase and oxidative phosphorylation (Staples *et al*., 2004). Using thermal imaging Volynchik *et al*. (2006) found a hotspot in the middle of the protothorax, thought to be a thermoregulatory centre, with a decreasing heat gradient to the extremities.

The metabolic source of heat generation was identified as substrate cycling between fructose-6-phosphate and fructose diphospate by Newsholme *et al*. (1972). This was further supported by the fact that honeybees that are unable to forage in cold temperatures lack fructose diphosphate activity. However, this hypothesis has been disputed by Staples *et al*. (2004), as when studying *Bombus rufocinctus* Cresson, 1863 (Hymenoptera: Apidae), heat generation as a result of fructose diphosphate was found to be less than 7% of the heat required on a cold day. The thoracic temperature of *Bombus wilmattae* Say (Hymenoptera: Apidae) was found by Nieh *et al*. (2006) to be elevated in individuals collecting nectar with a high sucrose concentration. The average thoracic temperature increased by 4.2˚C for every $1 mol^{-1}$ increase in sucrose concentration. The authors suggest this is the result of a strategy to collect 'high value' food and return it to the nest quickly.

Thermoregulation within the colony

Bumblebee colonies exhibit social homeostasis (Emerson, 1956) to ensure optimum conditions are maintained for brood development. When establishing a colony, queens incubate eggs throughout their development with abdominal heat, maintaining a brood temperature between 30 and 32˚C (Heinrich, 1975). After their emergence, workers take over all thermoregulatory roles (Goulson, 2010) and maintain colony homeostasis. Bumblebee colonies maintain their temperature to a set point between 27 and 33˚C (Vogt, 1986a), responding to high and low temperature fluctuations with wing fanning and heat generation respectively (Weidenmüller *et al*., 2002, Weidenmüller, 2004).

Weidenmüller (2004) found workers differed in their response to a temperature change in the nest, with the threshold of fanning initiation and the duration of fanning varying between individuals. This resulted in an increased number of fanning individuals at extreme high temperatures, compared to moderately high. The same response was recorded with increased CO_2 levels. Smaller colonies (below 60 workers) were found by Weidenmüller *et al*. (2002) to be less able to maintain a constant temperature in fluctuating environmental conditions. It follows, therefore that smaller colonies are more likely to fail as a result of extreme environmental temperatures.

1.11. *BOMBUS TERRESTRIS*: SUBSPECIES

Bombus terrestris can be further sub-divided into nine subspecies (Figure 1.10, Rasmont *et al*., 2008). *B. t. audax* is native to the United Kingdom and the Republic of Ireland and can be distinguished by a narrow yellow collar, mixed with black (Rasmont *et al*., 2008). The bumblebee primarily used in commercial colonies *B. t. dalmatinus* (Ings *et al*, 2005a), can be distinguished by its wide yellow collar, with an absence of black (Rasmont *et al*., 2008), with its native range including South East France, Northern Italy, the Balkanic Peninsulas, Turkey and North Iran, (Rasmont *et al*., 2008). Other subspecies include *B. t. africanus* Krüger, 1956, native to North Africa, *B. t. calabricus* Krüger 1958, native to Southern Italy and Sicily, *B. t. canariensis* Pérez 1895 native to, and exclusively inhabiting the Canary Islands, *B. t. lusitanicus* Krüger 1956 native to South West France, the Iberian Peninsula, Balearic Islands and Madeira, *B. t. sassaricus* Tournier 1890 native to Sardinia and *B. t. xanthopus* Kriechbaumer 1870, native to Corsica, Capraia Island and Elba Island (Rasmont *et al*., 2008).

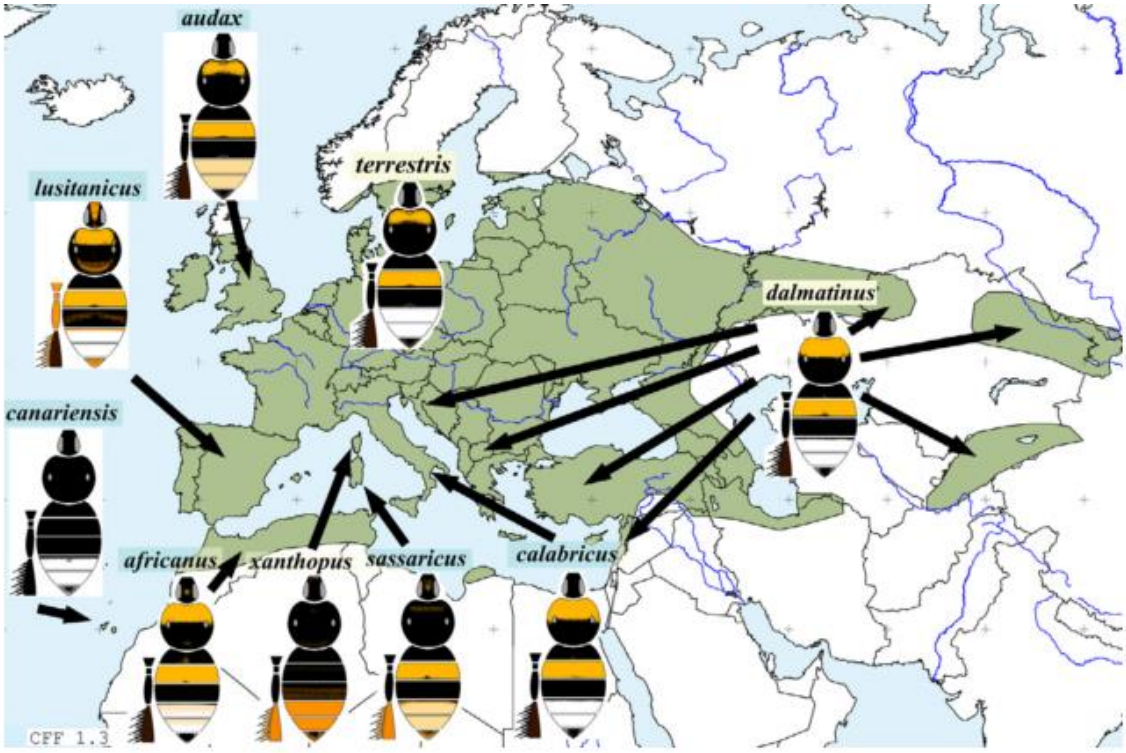

Figure 1.10 Figure and legend taken from Rasmont *et al*. (2008). Natural distribution of *Bombus terrestris* subspecies. The shaded area indicates the range over which *B. terrestris* occurs and the arrows point to the approximate centres of distribution of the nine subspecies.

Life cycles vary between subspecies, with those inhabiting northern regions, such as *B. t. audax*, typically having a winter diapause and only one colony cycle per year (univoltinism) (Rasmont *et al*., 2008). However, Mediterranean subspecies are known to have many cycles per year (multivoltinism; Rasmont *et al*., 2005), often with a brief period of summer aestivation (Rasmont *et al*., 2008).

1.12. *BOMBUS TERRESTRIS* IN THE UK: *B. T. AUDAX* AND *B. T. DALMATINUS*

Despite the presence of the UK-native *B. t. audax*, the commercial importation *B. t. dalmatinus* has proceeded in large numbers, without regulation (Ings *et al*., 2005a). Non-native bees were originally used in glasshouses, but have inevitably escaped and initiated wild populations (Ings *et al*., 2006). Ings *et al*. (2005b) were the first to highlight the potential problems arising from these imported bees, and found significant differences in nectar foraging performance between subspecies. They highlight an array of detrimental effects associated with the establishment of non-native bumblebees, including changes in plant seed set due to differential pollination, the risk of hybridisation between bee subspecies, and possible loss of genetic variability, as well as the introduction of parasites and pathogens.

B. t. dalmatinus is the chosen subspecies for use in commercial colonies, due to its large worker size and the high success rate and size of their colonies (Velthuis and van Doorn, 2006). However these characteristics make them hazardous to import as they can facilitate the competitive displacement of the native subspecies, especially heightened in areas of sparse floral resources (Goulson, 2003; Ings *et al*., 2005b). Ings *et al*. (2006) found consistently higher nectar foraging rates (1.6 times) in *B. t. dalmatinus*, the result of a larger worker size, and an increased production of queens when compared to the native *B. t. audax*. Subspecies were also found to differ in their floral colour preferences and their learning abilities (for example, during foraging), with *B. t. dalmatinus* more responsive to negative feedback (Ings *et al*., 2005a). In mating choice experiments, 71% of commercial bees preferred to mate with their own subspecies, however, mating with native bees did occur (Ings *et al*., 2005a). This

highlights the possibility of hybridisation between subspecies, an occurrence that may result in maladaptive genes and a loss of genetic diversity (Ings *et al*., 2005a). Given the scale of *B. t. dalmatinus'* importation into the UK (10,000 annually) and the ability of the species to naturalise easily (Ings *et al*., 2005b) it is highly likely that *B. t. dalmatinus* has already established in the UK (Ings *et al*., 2006).

1.13. **WINTER ACTIVE BUMBLEBEES**

The typical life cycle of *Bombus terrestris* in Northern Europe consists of a period of activity from spring to autumn and a period of winter diapause, with one colony cycle per year (as described in previous sections). However, Stelzer *et al*. (2010) and numerous anecdotal reports have reported sightings of *B. t. audax* workers, queens and males in winter, suggesting the presence of winter active colonies. Stelzer *et al*. (2010) suggest winter flowering plants such as *Mahonia* spp Nutt. (Ranunculales: Berberidaceae), present in urban parks, can provide a rich source of winter forage to sustain colonies over winter. Additionally, climate change, urban heat islands, and urban gardens may provide improved foraging conditions to support pollen and nectar collection. Stelzer *et al*. (2010) also speculate that hybridisation with *B. t. dalmatinus*, a non-native commercial subspecies with two natural generations, may be the cause of the winter activity.

Due to their extensive role in commercial pollination, bumblebees are being exposed to increasing levels of pesticides (discussed in later sections), leading to concerns being raised as

to the impacts on colony health and longevity (Potts *et al*., 2010a). These issues are critical to the continued use of bumblebees as commercial pollinators, and need to be viewed in conjunction with measures of temperature stress.

1.14. PESTICIDES AND AGRICULTURE

A pesticide, in the context of crop protection, can be defined as any compound used to destroy or repel pests (Zhang *et al*., 2011). Pesticides mitigate the effects of crop damage, including the negative effects of disease, weed growth and insect herbivory (Matthews *et al*., 2014). Given the growth of the human population, pesticides have been increasingly used to maintain yields of commercially valuable food crops (Oerke and Dehne 2004; Zhang, 2011; Tscharntke *et al*., 2012) including cereals, soybeans and maize, (Blacquière *et al*., 2012). Globally, there are 9,000 species of insect pest known to cause crop damage, which result in a 14% loss in yield (Oerke and Dehne 2004; Pimentel, 2009; Zhang *et al*., 2011).

Historically, a wide range of pesticides has been available, including organochlorides, carbamates, pyrethroids, herbicides and fungicides (Aktar *et al*., 2009). Many pesticides, such as the organochloride DDT, have been subsequently banned due to detrimental impact on humans and the environment (Aktar *et al*., 2009). Most recently, neonicotinoids have been used to combat crop pests, preserving up to one fifth of a crop yield from pest damage (Oerke and Dehne 2004; Matthews *et al*., 2014). Neonicotinoids can be applied as a seed coating or a foliar spray (Matthews *et al*., 2014). Compared to seed treatments, the concentration of

pesticides applied to a plant as a result of a foliar spray is highly variable (Matthews *et al*., 2014). Foliar sprays suffer from runoff; leaching and spray drift (Cryer and Applequist, 2003) and may collect in the plant's nectar reservoirs if directly sprayed on to flowers. However, a number of neonicotinoids such as thiacloprid are still available for purchase and application as commercial sprays, and domestic horticultural products (Table 1.1). Although thiacloprid lacks the same level of toxicity as pesticides from the N-nitroguanidine group, their toxicity to pollinators has been confirmed in several studies (Iwasa *et al*., 2004; Laurino *et al*., 2011). Concerns have been growing regarding the impact of neonicotinoids on non-target organisms such as pollinators; a topic explored further in the following sections.

1.15. NEONICOTINOIDS

Neonicotinoids are a class of nicotinic acetylcholine receptor agonists (nAChRs; Goulson, 2013) which are highly neurotoxic to insects (van der Sluijs *et al*., 2013). They mimic the acetylcholine neurotransmitter in the post-synaptic membrane of insect central nervous systems (Goulson, 2013) and bind with high affinity (van der Sluijs *et al*., 2013; Tomizawa and Casida, 2003). Neonicotinoids are now the most widely used class of insecticide in the world (Goulson, 2013), licenced to control pest species (Blacquière *et al*., 2012) on 140 crops and many horticultural products, generating $1 billion in sales in 2009 (Stoksad, 2013).

The main neonicotinoids used are imidacloprid, thiacloprid, thiamethoxam, clothianidin, dinotefuran, acetamiprid, nitenpyram and sulfoxaflor (van der Sluijs *et al*., 2013; Casida,

2010; Liu *et al*., 2008; Cutler *et al*., 2013). These can be partitioned into three groups; the N-nitroguanidines (imidacloprid, thiamethoxam, clothianidin and dinotefuran), nitromethylenes (nitenpyram) and N-cyanoamidines (acetamiprid and thiacloprid) (Jeschke *et al*., 2011; Goulson, 2013). The N-nitroguanidines are recognised to be more toxic to arthropods, with the addition of a nitro-containing group, as opposed to a cyano group N-cyanoamidines (Blacquière *et al*., 2012; Iwasa *et al*., 2004; Laurino *et al*., 2011), as a result of the fast bio-transformation of the N-cyanoamidines (Blacquière *et al*., 2012; Brunet *et al*., 2005). Of the neonicotinoid group, imidacloprid is the most popular, being the second most widely used insecticide in the world (Pollak, 2011; Feltham *et al*., 2014). Its growth in the UK has been rapid (Figure 1.11).

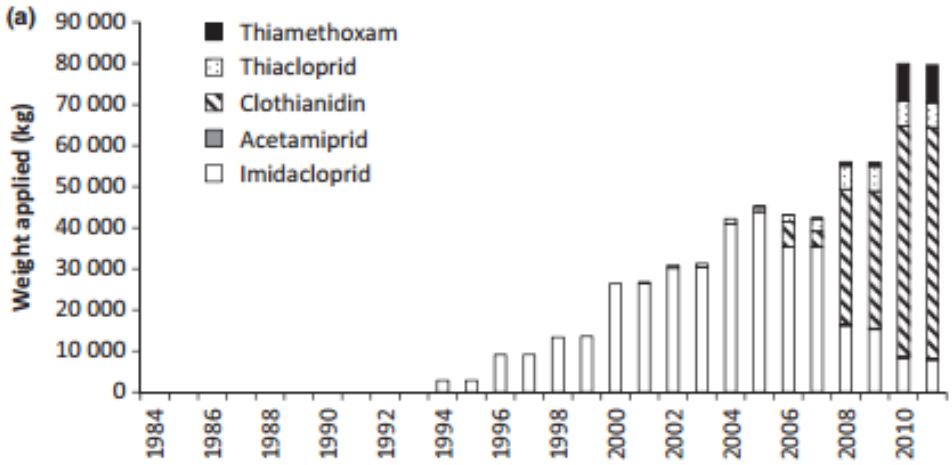

Figure 1.11 Figure and legend taken from Goulson (2003). a) Annual usage (kg) of neonicotinoids in agriculture and horticulture in the UK, one of few countries from which detailed records are available (Defra, 2012). Note that these figures do not include garden or amenity use, or use from treatment of pets. In 2011, the area of land treated was approximately 1.3 million ha.

Imidacloprid is used, primarily in seed treatment form (Goulson, 2013) in the protection of cereals, oilseed rape, corn, cotton, sunflower and sugar beets (Elbert *et al*., 2008; Whitehorn *et al*., 2012) from sucking insects (Tomizawa and Casida, 2003). Thiacloprid is used in the protection of fruit crops, for example raspberries, and is often used in a spray form (Lye *et al*., 2011; Goulson, 2013).

1.16. NEGATIVE IMPACTS OF NEONICOTINOIDS

Shortly after the introduction of neonicotinoids to agricultural ecosystems, a range of disorders was reported in honeybee colonies and individuals, including abnormal foraging, disease sensitivity, queen loss and colony collapse (van der Sluijs *et al*., 2013). A wealth of evidence has been growing, suggesting a link between the use of neonicotinoids in crop management and the global decline of bees, including bumblebees (Blacquière *et al*., 2012; Whitehorn *et al*., 2012).

Managed honeybees and wild pollinators are exposed to neonicotinoids when foraging on both wild flowers and managed crops (Goulson, 2013). Oilseed rape, sunflower and maize provide a substantial amount of forage for pollinators, however, seeds are routinely coated with neonicotinoids, which are systemically distributed throughout plant tissues, including pollen and nectar (Goulson, 2013). Given this fact, and that neonicotinoids persist in soils (van der Sluijs *et al*., 2013), they can often be transferred to wildflowers in field margins, further contaminating potential pollinator forage (Whitehorn *et al*., 2012; Goulson, 2013).

Krupke *et al*. (2012), for example, found neonicotinoid concentrations of up 9 parts per million (ppm) on wild flowers growing near treated crops. Sunflowers treated with 0.7mg ^{14}C-imidacloprid in the form of a seed coating, resulted in concentrations of 3.9 ± 1.0μgkg^{-1} in pollen and 1.9 ± 1.0μgkg^{-1} in nectar (Schmuck *et al*., 2001). Imidacloprid metabolites such as imidacloprid olefin, are also toxic to insects but their presence is often not recorded (Goulson, 2013).

As a result of the negative impacts of neonicotinoids, the European Food Safety Authority (EFSA) commissioned an evaluation of the impacts of three neonicotinoids, clothianidin, imidacloprid and thiamethoxam, used as seed treatments, on honeybee health (European Food Safety Authority, 2013). Routes of exposure investigated were via pollen and nectar, dust exposure and exposure through guttation fluid. The assessment included a risk assessment of acute and chronic exposure to neonicotinoids and effects on larval development, behaviour and sub-lethal effects (European Food Safety Authority, 2013). As a result of the study, it was concluded that the neonicotinoids tested should only be used on crops not attractive to honeybees. Results could not be extrapolated to predict the effects of neonicotinoids on bumblebees due to their differing biology and nesting habits. For example, bumblebees may come into increased contact with soluble neonicotinoids in groundwater as a result of subterranean nesting.

Due to the increasing evidence suggesting the negative impact of pesticides on bees, the European Commission imposed a two year ban, as of December 2013, on the planting of

seeds treated with clothianidin, imidacloprid and thiamethoxam (Stoksad, 2013). Sprays containing these neonicotinoids were also banned for use on crops providing bee forage. However, a number of neonicotinoids such as thiacloprid are still available for purchase and application as commercial sprays, and domestic and horticultural products (Table 1.1).

1.17. THIACLOPRID

Thiacloprid was developed for use by Bayer Crop Science, with a conditional registration issued on September 26th 2003 (United States Environmental Protection Agency, 2003). It is known by the trade name of Calypso; YRC-2894, with a generic name of [3-[(6-chloro-3-pyridinyl)methyl]-2- thiazolidinylidene]cyanamide. It is part of the neonicotinoid class, sub-group chloronicotinoid, and acts, as with other neonicotinoids, as an acetyl choline receptor antagonist (United States Environmental Protection Agency, 2003).

According to the registration documentation (United States Environmental Protection Agency, 2003), core toxicity tests indicated that thiacloprid was not likely to affect bee health. The document reports that thiacloprid toxicity is 'unlike other neonicotinoid insecticides' which 'have demonstrated very high to high acute toxicity to bees.' Thiacloprid is reportedly effective against aphids, thrips, whitefly and stemborer and is 'safe for bees' (Bayer Crop Science, 2014). A study by Elbert *et al*. (2000) suggests thiacloprid is suitable for use during the blossom period of bee-attractive crops due to its 'bee safety'.

Thiacloprid is available for both commercial and domestic use. Several products that are available, from retailers, to unlicensed consumers are listed in Table 1.1.

Table 1.1 Readily available neonicontinoid products. Adapted from The Soil Association (2013).

Product	Neonicotinoid	Stockists
Provado Vine Weevil Killer	Thiacloprid	B&Q Homebase Wilkinson
Provado Ultimate Bug Killer Spray	Thiacloprid	Wilkinson Homebase
Provado Ultimate Bug Killer Ready to Use	Thiacloprid	Wilkinson Homebase
Provado Ultimate Bug Killer Concentrate	Thiacloprid	Wilkinson Homebase

Although the United States Environmental Protection Agency report (2003) and Bayer Crop Science (2014) reports thiacloprid is 'safe for bees', a number of studies have found evidence that suggest the contrary. In a study by Vidau *et al*. (2011), exposure to sublethal concentrations of thiacloprid (5.1ppm, corresponding to $1/100^{th}$ of the LD_{50}) resulted in increased mortality in honeybees previously infected by the parasite *Nosema ceranae* n. sp. (Microspora, Nosematidae). Thiacloprid was also found to have a greater mortality rate than controls in oral toxicity tests in starved honeybees, in a study by Laurino *et al*. (2011). The LD_{50} of thiacloprid was found by Iwasa *et al*. (2004) to be $14.6 mgbee^{-1}$. In tests by Skerl *et al*. (2009), honeybees foraging in apple orchards treated with thiacloprid were found to collect contaminated pollen and bring it back to the colony. Levels were not high enough to induce

mortality (0.3ppm), however, the authors suggest that sub-lethal effects could be present, given that contaminated pollen would be fed to the honeybee brood and may affect future colony development.

There is currently no evidence surrounding the sub-lethal effects of thiacloprid on bumblebees and, as the European Food Safety Authority (2013) report suggests, this represents a significant knowledge gap. Additionally, there have been no studies on the effects of any pesticide on activity thresholds, a topic investigated in this thesis. Chapter 6 aims to address this knowledge gap, with thermal activity thresholds monitored, after bees were exposed to sub-lethal concentrations of a domestic thiacloprid product.

1.18. THESIS STRUCTURE

CHAPTER 2 - CAN WINTER-ACTIVE BUMBLEBEES SURVIVE THE COLD? ASSESSING THE COLD TOLERANCE OF BOMBUS TERRESTRIS AUDAX AND THE EFFECTS OF POLLEN FEEDING

Winter activity brings a novel set of thermal challenges to bumblebee workers. This chapter assesses the ability of workers to withstand acute and chronic cold stress, using a range of cold tolerance indices. The influence of diet and the capacity of workers to undergo rapid cold hardening is also investigated. This chapter identifies, for the first time, the cold tolerance profile of the British bumblebee *B. t. audax*, in addition to the first evidence of RCH in *Hymenoptera*, and informs its capacity for continued winter activity. This chapter has been published in PLOS ONE.

CHAPTER 3 - CAN NON-NATIVE BUMBLEBEES SURVIVE UK WINTERS? ESTABLISHMENT RISK OF THE COMMERCIALLY-IMPORTED BUMBLEBEE BOMBUS TERRESTRIS

Non-native bumblebees (*Bombus terrestris dalmatinus*) have been exported to the UK in large numbers for the purposes of commercial pollination. Concern is growing regarding the establishment of the subspecies in the UK. This chapter assesses the ability of *B. t. dalmatinus* workers to withstand acute and chronic cold stress, using a range of cold tolerance indices. The capacity of workers to undergo rapid cold hardening is also investigated. This chapter identifies, for the first time, the cold tolerance profile of the non-native bumblebee *B. t. dalmatinus* and informs the likelihood of winter survival and establishment of this subspecies. Results are compared to those of *B. t. audax*, detailed in Chapter 2.

CHAPTER 4 - BUMBLEBEE ACTIVITY IN A CHANGING CLIMATE: THERMAL ACTIVITY THRESHOLDS OF NATIVE (BOMBUS TERRESTRIS AUDAX) AND NON-NATIVE (BOMBUS TERRESTRIS DALMATINUS) BUMBLEBEES

Chapters 2 and 3 detail the thermal limits to survival in native and non-native bumblebees. In order to sustain colonies, bumblebees must not only survive, but remain active at unfavourable temperatures to facilitate foraging. This chapter examines the thermal limits to co-ordinated activity in bumblebee workers of two subspecies (*B. t. audax* and *B. t. dalmatinus*), using well-established lab indices. This will provide information on the capacity of bumblebees to remain active, and therefore foraging, in an era of climate change and winter-active bumblebees.

CHAPTER 5 – SEASONAL PHENOLOGY OF WILD B. T. AUDAX IN BIRMINGHAM AND THE CAPACITY FOR WINTER COLONY SURVIVAL

This chapter aims to contextualise lab indices of activity and cold tolerance by observing wild and managed bumblebees in a field setting. First, transect walks were regularly conducted at a botanical garden in Birmingham, over a period of 27 months, to identify winter-active bumblebee workers. Second, commercial colonies were housed in a field setting at the beginning and end of winter to determine the capacity for bumblebee workers to remain active in sub-optimal temperatures. This chapter reflects protocols undertaken by Stelzer *et al*. (2010) in London and aims to identify if winter-active bumblebees are able to survive in a botanical garden in Birmingham.

CHAPTER 6 – THE EFFECT OF THE NEONICOTINOID THIACLOPRID ON THE SURVIVAL AND THERMAL ACTIVITY THRESHOLDS OF BOMBUS TERRESTRIS AUDAX AND B. T. DALMATINUS

Currently, there exists an intense scientific debate over the impact of neonicotinoids on beneficial pollinators. Although field concentrations of neonicotinoids often do not cause mortality, increasing evidence has highlighted the detrimental impact of sub-lethal concentrations and the resulting impacts on behaviour and foraging. In this chapter, I investigate the impact of a neonicotinoid, thiacloprid, on bumblebee thermal activity thresholds (comparable to those in Chapter 4). This is the first time the effect of pesticides has been investigated on activity thresholds and results will determine if neonicotinoids compromise bumblebee activity at sub-optimal temperatures.

CHAPTER 7 - GENERAL DISCUSSION

This chapter draws together the findings of the above chapters and discusses them in the light of recent evidence. Further avenues for research are also discussed.

1.19. **THESIS AIMS**

The overall aim of this project is to identify the cold tolerance and activity thresholds of native (*Bombus terrestris audax*) and non-native (*Bombus terrestris dalmatinus*) bumblebees. This data will be used to determine if the subspecies are able to survive UKs winters. The specific aims of the project are:

1. To determine the cold tolerance of *B. t. audax* and *B. t. dalmatinus* using laboratory methods.
2. To establish if *B. t. audax* and *B. t. dalmatinus* are able to undergo Rapid Cold Hardening.
3. To determine the activity thresholds of *B. t. audax* and *B. t. dalmatinus* and assess their ability to acclimate.
4. To assess the impact of diet and pesticides on *B. t. audax* at low temperatures.
5. To conduct field experiments to determine the abundance and survival of wild and managed *B. t. audax* in winter.

CHAPTER 2

CAN WINTER-ACTIVE BUMBLEBEES SURVIVE THE COLD? ASSESSING THE COLD TOLERANCE OF *BOMBUS TERRESTRIS AUDAX* AND THE EFFECTS OF POLLEN FEEDING

2.1. ABSTRACT

There is now considerable evidence that climate change is disrupting the phenology of key pollinator species. The recently reported UK winter activity of the bumblebee *Bombus terrestris* brings a novel set of thermal challenges to bumblebee workers that would typically only be exposed to summer conditions. Here I assess the ability of workers to survive acute and chronic cold stress (via lower lethal temperatures and lower lethal times at 0°C), the capacity for rapid cold hardening (RCH) and the influence of diet (pollen versus nectar consumption) on supercooling points (SCP). Comparisons are made with chronic cold stress indices and SCPs in queen bumblebees. Results showed worker bees were able to survive acute temperatures likely to be experienced in a mild winter, with queens significantly more tolerant to chronic cold temperature stress. The first evidence of RCH in any Hymenoptera is shown. In addition, dietary manipulation indicated the consumption of pollen significantly increased SCP temperature. These results are discussed in the light of winter active bumblebees and climate change.

2.2. INTRODUCTION

Climate change has resulted in changes to the physiology, survival, abundance and range of many organisms (Parmesan, 2006). Characterisation of these changes is essential to understanding and mitigating the potential impacts of climate warming (Deutsch *et al*., 2008) and the provision of vital ecosystem services. This is particularly important in insect pollination (Hillstrom *et al*., 2008; Cornelissen, 2011), with considerable evidence reporting phenology shifts in key pollinator species (Kremen *et al*., 2007; Memmott *et al*., 2007; Roberts *et al*., 2011). Spring advancement, extended growing seasons, milder winters and the availability of winter forage (Parmesan, 2006) have all combined to allow some normally univoltine species to become bivoltine, as evidenced by winter active bumblebees, *Bombus terrestris*, in the southern UK (Stelzer *et al*., 2010). This raises a number of new challenges, in particular the survival of life stages previously unexposed to winter conditions.

At temperate latitudes, a specialised state of dormancy, termed diapause, is typically utilised as an overwintering strategy in virtually all insects (Denlinger, 2002; Bale and Hayward, 2010) and winter survival of cold stress outside of diapause is greatly reduced (Rinehart *et al*., 2006). In bumblebees, as with many bee species, it is only mated females (queens) that enter diapause and overwinter, but we know very little about how bumblebee queens or other life stages (workers and males) cope with cold conditions. Diapause in bumblebees is not thought to be obligate, as there is evidence that commercial colonies can be established by queens without diapause (Tasei, 1994). There are also several examples of bivoltinism in Mediterranean field populations (Estoup *et al*., 1996; Rasmont *et al*., 2005), as well as in New

Zealand and Tasmania (Buttermore, 1997). In addition, non-diapause characteristics can be artificially selected for, and can persist across multiple generations (Beekman *et al*., 1999). Considerable flexibility also exists in the seasonal timing of diapause in *B. terrestris*, with both a summer aestivation and winter hibernation reported in different regions of Turkey (Gurel *et al*., 2008). The issue in the UK, and in other parts of Northern Europe, is that *B. terrestris* queens appear to be averting diapause, or have a greatly curtailed diapause, under warmer conditions and so are establishing new colonies in late summer and autumn (Stelzer *et al*., 2010). This is in stark contrast to a previous study (Alford, 1969), that reported a period of dormancy spanning 6–9 months in the UK.

Certainly to assume climate change will reduce cold-induced winter mortality is over simplistic. Indeed there is increasing evidence of more frequent extreme climatic events (Rosenzweig *et al*., 2001). Key to the winter survival of insects is the magnitude, frequency and timing of cold events with variable thermal regimes conferring a reduction in chill-injury and increased longevity compared to constant low temperatures (Koštál *et al*., 2007). Thus, it is crucial to determine whether key pollinator species such as bumblebees will benefit from climate change, or if winter activity might have a negative impact on their abundance, distribution and pollination service provision. Addressing this knowledge gap is important from both conservation and food security perspectives, given the critical pollination services provided by bumblebees (Klein *et al*., 2007). *Bombus terrestris*, in particular, is a key economic crop pollinators, essential in the pollination of vegetable, fruit and seed crops (Klein *et al*., 2007) and is responsible for the annual production of 40,000ha of tomato crops

(*Solanum lycopersicum*) (Velthuis and van Doorn, 2006). As a result of their success, commercially-produced bumblebee colonies are exported from Europe at a rate of 850,000 per year to Asia, North Africa, Chile, New Zealand and the Middle East (Velthuis and van Doorn, 2004).

To survive winter, active colonies of the UK subspecies, *B. t. audax*, must first be able to withstand potential acute low temperatures when foraging, and chronic cold exposures within the colony. Bumblebee colonies are known to regulate their temperature to a set point between 27 and 33°C (Vogt 1986a). However, chronic low temperatures have been found to disrupt colony thermoregulation and have a negative impact on colony fecundity and brood incubation temperature (Vogt 1986b). With a reduced number of workers, smaller colonies are less able to respond to environmental stresses such as temperature fluctuations (Weidenmüller *et al*., 2002). Acute cold exposure also poses a risk to individual bumblebees foraging in low daytime temperatures, or bees spending the night away from the nest (Free, 1955; Spaethe and Weidenmüller, 2002). The response of workers to chronic and acute cold exposure is currently unknown, presenting an uncertain risk to the survival of winter-active colonies, and thus pollinator abundance the following spring.

Insects employ several mechanisms to increase their tolerance of chilling or cold shock, one of which is Rapid Cold Hardening (RCH). This is where a short period of acclimation (for example, 1 hour) leads to significantly higher survival at sub-zero temperatures (Lee *et al*., 2006). The ability to rapidly cold harden was first identified in the flesh fly (*Sarcophaga*

crassipalpis) (Chen *et al*., 1987). It has since been identified in 8 orders and 26 families; however, RCH has never been recorded in Hymenoptera (Denlinger, 2010). In the case of winter-active *B. t. audax*, RCH is likely to have considerable adaptive significance in the survival of workers and active queens.

Gut contents are also known to play an important role in the cold tolerance of insects and their manipulation is often a discriminating factor between freeze tolerant and freeze avoiding strategies (Bale, 1996). Freeze avoiding (FA) insects seek to reduce their freezing temperature/supercooling point (SCP), and are known to evacuate their guts to exclude ice nucleating agents (INAs) such as bacteria, dust and food particles (Duman and Patterson, 1978) which might promote the formation of ice crystals (Zachariassen, 1982). Typical SCPs for FA insects are below -10°C (Denlinger, 2010) *e.g*. *Collembola* spp. (Insecta: Collembola) with SCPs between -15 and -35°C (Lee, 1989). However, freeze tolerant species actively produce INAs to facilitate more controlled freezing at higher sub-zero temperatures, and so typically have SCPs between -5 and -10°C *e.g*. overwintering larvae of a stem-galling tephritid fly *Eurosta solidaginis* (Diptera, Tephritidae), with an SCP of -9°C (Bale *et al*., 1989). Certainly gut contents can have a huge impact on an organism's SCP, and consequently the winter survival of FA species. For example, the absence of gut contents in starved, winter Antarctic microarthropods is responsible for a shift in SCP from a summer 'high' group (mean SCP -7°C) to a winter 'low' group (mean SCP -25°C) (Worland and Convey, 2001). It is not known if *B. terrestris* is FT or FA, but we hypothesise this species will not tolerate freezing, in common with several other bee species, *e.g*. *Osmia cornuta* and *O. rufa*

(Hymenoptera: Megachilidae), both with SCPs typically below -24°C (Krunić and Stanisavljevic, 2007) and *Megachile rotundata* with SCPs of -8°C (Sheffield, 2008). Given the ice nucleating properties of pollen (Diehl *et al*., 2001) winter active workers, especially those which do not return to the colony at night (Alford, 1969), are at increased risk of mortality due to freezing. Winter active bumblebees (as described by Stelzer *et al*., 2010) forage for both nectar and pollen in order to provision their colonies. Currently, however, there does not appear to be any direct evidence that workers ingest pollen. If they do, this could negatively impact their survival at sub-zero temperatures. For example, the presence and quantity of ice nucleators in the gut was found to impact cold tolerance in *M. rotundata* (Krunić, 1971).

The objectives of this study were to assess the ability of *B. t. audax* workers to tolerate UK winter temperatures and compare their cold tolerance with active queens. This involved a determination of their capacity for RCH and survival following prolonged periods of cold exposure consistent with what might be experienced within winter colonies. An assessment of their freezing temperature (SCP) and post-freezing survival also permitted classification of this species as either 'freeze tolerant' or 'freeze avoiding', consistent with criteria outlined by Bale (1996). Finally, the impact of diet on cold tolerance was investigated, to determine the effect of pollen on the cold tolerance of foraging bumblebees.

2.3. METHODS

Culture system.

Mature colonies of *B. t. audax*, were obtained from Biobest NV (Westerlo, Belgium), maintained at 20°C in constant darkness and manipulated under red illumination to minimise disturbance (Sadd, 2011). Nectar was available within the colony using a wick system connected to a reservoir of BioGluc® nectar and pollen paste was available *ad libitum* (Biobest NV). Mated, non-diapausing queens were also provided by Biobest NV (Westerlo, Belgium) and were individually housed in 'feeding boxes' containing pollen and nectar *ad libitum* (unless otherwise stated). For each experimental treatment where survival was assessed, 6 replicates of 5 bees were used, unless otherwise stated. Control samples of 6 replicates of 5 bees were exposed to 15°C for the maximum experimental duration and survival, determined as the response to gentle manipulation, was assessed after 72 h.

Lethal temperature

Bumblebees were placed into 6 test tubes (n=5 per tube) and secured with a foam bung. Bumblebee thoracic temperature was measured using Type K (exposed wire) thermocouples, connected to a thermocouple data logger (Pico® TC-08 Thermocouple Data Logger). Test tubes were placed into a programmable alcohol bath (Haake® Phoenix 11 P2, Thermo Electron Corporation), and were cooled from 20°C, at a rate of 0.2°Cmin^{-1} to a range of sub-zero temperatures (-5, -6, -7, -8 and -9°C). Bumblebees were held at each temperature for 15min before the temperature was increased back to 20°C at 0.2°Cmin^{-1}. Preliminary

experiments confirmed that all bees had equilibriated to the holding temperature within 15min. Bees were then transferred to rearing temperature and were housed in 'recovery boxes' in their experimental replicates (6 boxes, with 5 bees per box). Pollen and nectar was available *ad libitum* and survival was assessed after 72 h.

Lethal time at 0˚C

Bumblebee workers were placed in groups of 5 to 6 conical flasks (25ml Pyrex). Flasks were held at 10°C in a climate-controlled room for 1 hour to minimise cold shock prior to the experiment. Flasks were then transferred to a Fryka® incubator set at 0°C. A TinyTag® datalogger was added to the incubator to monitor temperature throughout the duration of the experiment. Preliminary experiments confirmed that all bees had equilibriated to the holding temperature within 15min. Bees were maintained in the incubator for a range of durations (2, 3, 5, 7, 10, 13 and 15 days) and were then transferred to a 10°C climate-controlled room for 1 hour to minimise cold shock after the experiment. Bees were then returned to their rearing temperature and were housed in 'recovery boxes' in their experimental replicates (6 boxes, with 5 bees per box). Pollen and nectar was available *ad libitum* and survival was assessed after 72 h. In experiments where bumblebee queens were used, queens were added in groups of 5 to 3 conical flasks (100ml Pyrex) and were manipulated in the same way as workers. Experimental durations of 5, 10, 15 and 20 days were used. Survival was assessed as previously described.

Rapid Cold Hardening

Determination of the discriminating treatment

Temperatures of -5 and -6°C were chosen as they represented the lowest sub-zero temperatures which induced mortality whilst having minimal incidence of freezing (supercooling point ranged from -5.0 to -10.9°C). Thirty bumblebees (6 replicates of *n*=5 bees per temperature) were taken from their rearing temperature (20°C), added to test tubes with thermocouples as previously described, and placed directly in an alcohol bath (Haake® Phoenix 11 P2, Thermo Electron Corporation) set at -5 or -6°C for a range of durations (2, 4, 8 and 10h). Bees were then re-warmed to rearing temperature at a rate of 0.2°Cmin^{-1} and survival was assessed after 72h. A treatment that resulted in 10–20% survival at a particular time interval was used as the discriminating treatment (Lee *et al*., 1987).

Rapid cold hardening response

Thirty bumblebees (6 replicates of 5 bees per treatment) were added to test tubes as previously described and exposed to one of two temperature regimes: 1h at 0°C before transfer to the discriminating treatment, re-warming at 0.2°Cmin^{-1}, or gradual cooling at 0.2°Cmin^{-1} to the discriminating treatment, and re-warming at the same rate. Survival in both experiments was assessed after 72h. RCH was detected as an increase in survival relative to direct transfer to the discriminating treatment.

Impact of rapid cold hardening on supercooling point

After a period of 1h at 0°C, the supercooling points (SCPs) of 30 workers were measured, using established methods (Hughes and Bale, 2009). Briefly, bees were inserted individually into test tubes containing type K exposed wire thermocouples, placed in an alcohol bath (Haake® Phoenix 11 P2, Thermo Electron Corporation) programmed to cool from 20°C to -20°C at a rate of 0.2°Cmin^{-1} and freezing exotherms were detected via a computerised recording system.

Impact of diet on gut pollen content and SCP

Pollen and nectar diet

Workers (*n*=30) and queens (*n*=21) were removed from their colonies and feeding boxes respectively and their SCPs measured as previously described.

Nectar-only diet

Workers were kept in 'feeding boxes' and fed nectar-only for a period of 3, 7 or 14 days before their SCPs were measured (*n*=30 per duration). Queens were kept individually in feeding boxes and fed nectar-only for 7 days (limited numbers meant only this time point could be tested), before their SCPs were measured (*n*=20). Following SCP measurement, worker bumblebees maintained under the feeding treatments described above were dissected and their gut contents examined under a high power microscope (*n*=16, 8 and 6 for 3, 7 and 14 day nectar-fed workers respectively). Gut contents were partitioned into 3µl aliquots, and

pollen grains were quantified in each sample. The total pollen count of each individual was then compared to the supercooling point of the bee and the days of nectar feeding.

***Statistical analysis*.**

A number of preliminary statistical tests were undertaken on the response variables to determine whether parametric or non-parametric testing could be employed. Normality was tested using Kolmogorov-Smirnov tests; equality of variance (homoscedasticity) was investigated using Levene's tests and time series were plotted to confirm the independence of samples. Pearson's Goodness of Fit tests were also undertaken on LTemp and LTime data to confirm the Probit models fitted the data. LTemp and LTime data were normally distributed and were subsequently analysed via Probit analysis in Minitab® (Finney, 1971). This was used to identify the temperature or time at which 10, 50 or 90% mortality occurred. All RCH and pollen consumption data were not normally distributed, but were homoscedastic and independent, therefore independent samples Kruskal Wallis tests with pairwise comparisons were undertaken using SPSS®. As multiple comparison tests in SPSS® include a correction for the familywise error rate, confidence interval bars on multiple comparison bar charts were adjusted using Bonferroni corrections (Figures 2.3, 2.4 and 2.5). RCH and SCP results were normally distributed, homoscedastic and independent and were analysed using a one way ANOVA in Minitab®. Fitted Line, linear regression was undertaken on total pollen versus supercooling point data. The model was checked using graphical analysis of residuals, including their normality, fit and order.

2.4. RESULTS

Lethal temperature

Lethal temperatures for 10, 50 and 90% mortality of workers were -5.0 ± 1.1, -7.8 ± 1.1 and -9.3 ± 1.1°C respectively (Figure 2.1). No mortality was recorded in control samples.

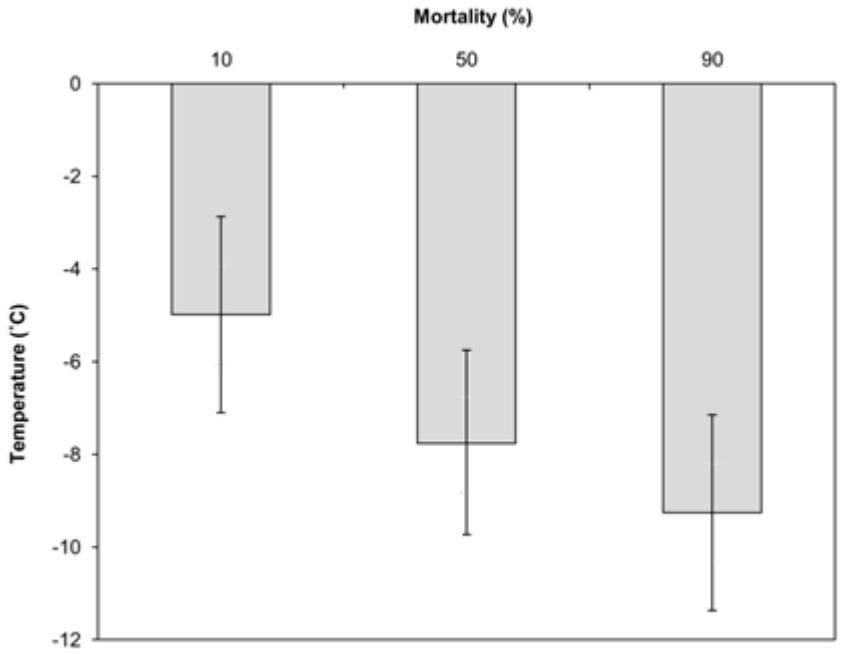

Figure 2.1. Lethal Temperatures of worker bumblebees (*Bombus terrestris audax*). The $LTemp_{10, 50}$ and $_{90}$ (±2SE) of worker bumblebees as determined by Probit analysis, *n*=30 per temperature.

Lethal time at 0°C

Lethal times for 10, 50 and 90% mortality at 0°C for workers were 2.3 ± 1.2, 7.2 ± 1.1 and 22.3 ± 1.2 days respectively (Figure 2.2). The $LTime_{50}$ for queens at 0°C was 25.6 ± 2.4 days, which was significantly different from that of workers (using non-overlapping fiducial limits; Hughes *et al*., 2009). As sample sizes were smaller for queens (*n*=15) than workers (*n*=30), only $LTime_{50}$ was determined. No mortality was recorded in control samples.

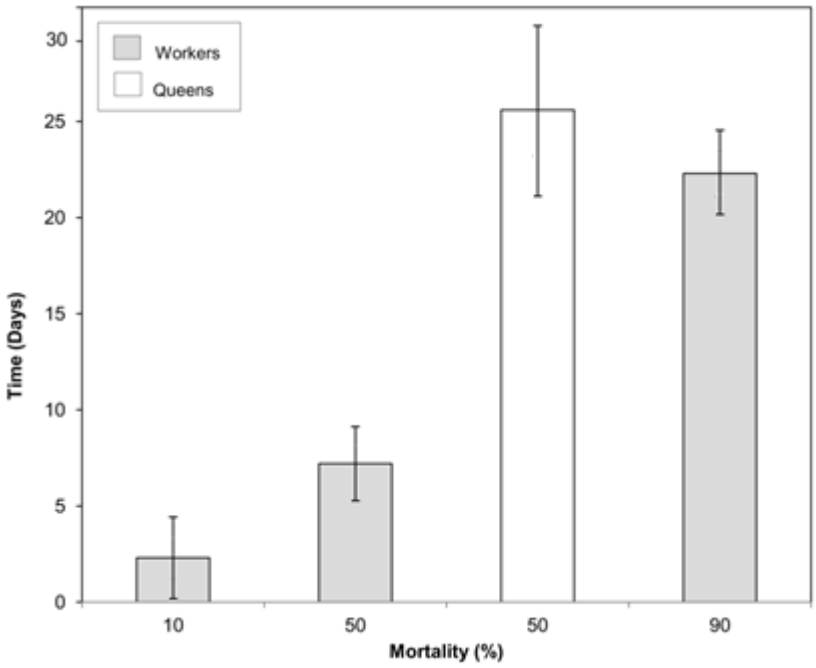

Figure 2.2. Lethal Times at 0°C of worker and queen bumblebees (*Bombus terrestris audax*). The $LTime_{10,\ 50}$ and $_{90}$ at 0°C (±2SE) of worker bumblebees as determined by Probit analysis, *n*=30 per duration.

Rapid Cold Hardening

Determination of the discriminating treatment

Mean survival of workers at -5°C decreased with increasing duration of cold exposure, from 80 ± 16.3% after 2h to 13.3 ± 13.3% after 10h exposure (Figure 2.3). Survival at 2 and 10h were significantly different (p=0.045 χ^2=9.92). Mean survival of workers at -6°C was consistently low (between 3.3 ± 3.3 and 16.7 ± 13.1% survival) with no significant difference between durations at this temperature (p=0.19, χ^2=.93). Based on the above data, 10h at -5°C was chosen to be the discriminating treatment to determine a RCH response.

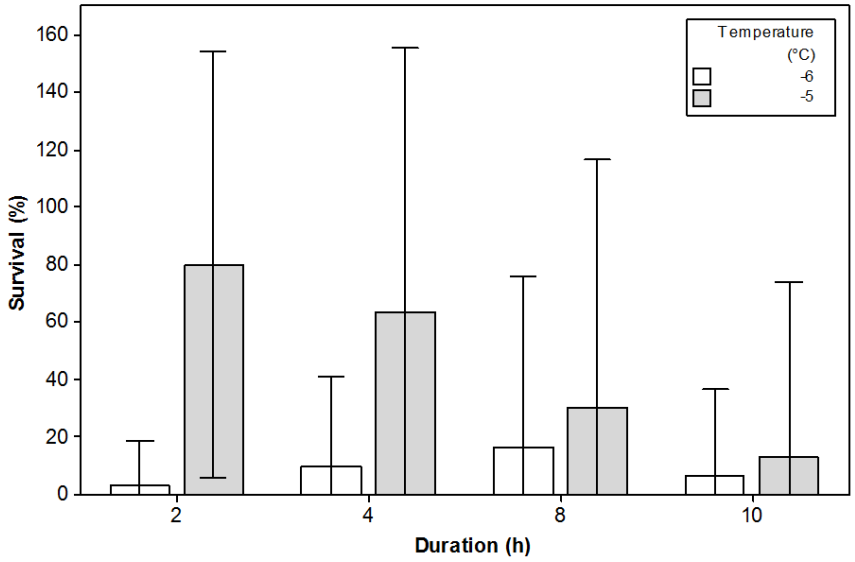

Figure 2.3 Determination of a discriminating treatment for rapid cold hardening in worker bumblebees (*Bombus terrestris audax*). Mean survival (with Bonferroni-corrected 95% confidence interval bars) of worker bumblebees exposed to periods of 2, 4, 8 and 10h at -5 and -6°C, n=30 per temperature.

Rapid cold hardening response

Survival of workers gradually cooled at 0.2°Cmin^{-1} to the discriminating treatment, (100 ± 0%) was significantly higher (p=0.002, χ^2=-13.17) than survival as a result of direct transfer (13.3 ± 13.3%; Figure 2.4). Survival following a 1h pretreatment at 0°C, before transfer to the discriminating treatment, was not significantly different to direct transfer to the discriminating treatment alone (p=0.08, χ^2=-8.67).

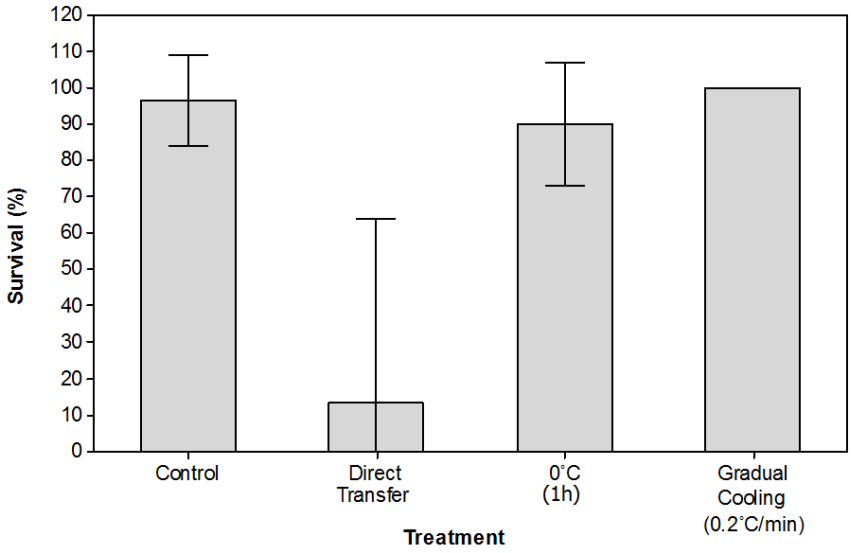

Figure 2.4 A rapid cold hardening response in worker bumblebees (*Bombus terrestris audax*). Mean survival (with error bars representing the 95% confidence interval for the mean, with a Bonferroni correction) of worker bumblebees after direct transfer to the discriminating treatment (-5°C for 10h), a pretreatment of 1h at 0°C before transfer to the discriminating treatment or gradual cooling (at 0.2°Cmin^{-1}) to the discriminating treatment. n=30 per treatment.

Rapid cold hardening and supercooling point

The supercooling points of workers exposed to a pretreatment of 8h at 0°C were not significantly different from controls (Table 2.1) (F=1.12, p=0.48).

Table 2.1 Supercooling points (SCP) of *Bombus terrestris audax* with no pretreatment (control) and a pretreatment a 0°C for 1h (RCH), *n*=30.

Treatment group	*n*	Mean ± SE (°C)	Range (°C)
SCP control	30	-7.1 ± 0.2	-5.0 to 10.9
SCP with RCH	30	-7.3 ± 0.2	-5.6 to -12.8

Impact of diet on gut pollen content and SCP

Preliminary experiments indicated both workers and queens suffered 100% mortality as a result of freezing. The mean SCP of workers decreased from -7.1 ± 0.2°C in controls to -9.7 ± 0.5, -11.7 ± 0.5 and -12.5 ± 0.5˚C after 3, 7 and 14 days of nectar-only feeding respectively (Figure 2.5). Each nectar-only feeding group had a significantly lower SCP than controls ($p \leq$ 0.01 in all cases). Only the 3 and 14 day groups were significantly different from each other (p=0.03, χ^2=37.57). The mean SCP of queens significantly decreased from -7.4 ± 0.3°C in controls to -10.6 ± 0.7°C after 7 days of nectar feeding (p=0.01 χ^2=50.58). The SCPs of workers and queens were never significantly different from each other (p>0.05).

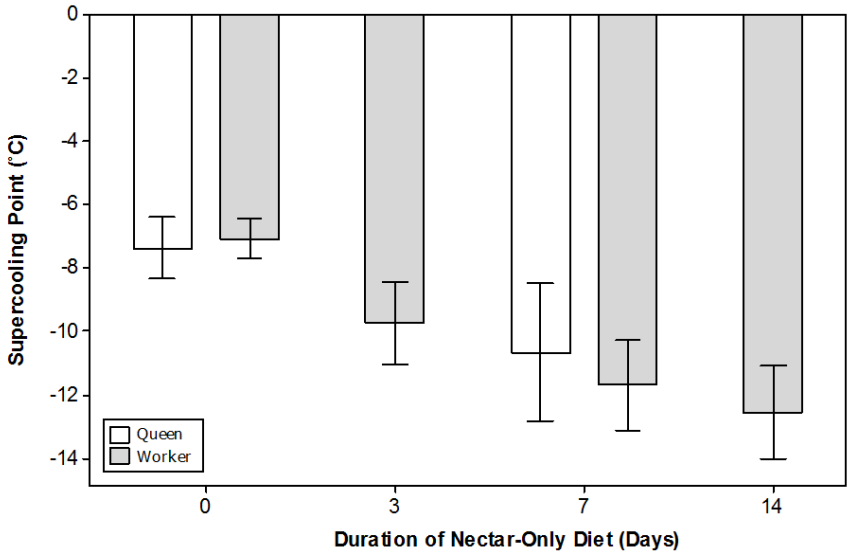

Figure 2.5 The effect of diet on supercooling point in queen and worker bumblebees (*Bombus terrestris audax*). Mean supercooling points (with Bonferroni-corrected 95% confidence interval bars) of *Bombus terrestris audax* workers and queens when fed a nectar-only diet for 3, 7 or 14 days with pollen and nectar-fed controls (n=30 for all worker treatments and n=20 and 19 for queen controls and 7 day nectar-fed-queens respectively).

There was a significant, linear relationship between the total number of pollen grains in the gut and the supercooling point of 7-day nectar-fed bees (n=8, p<0.02, R^2=65%), but not in 3-day or 14-day nectar-fed bees (Figure 2.6). There was also a significantly lower number of pollen grains in the guts of 14-day nectar fed bees compared to 7-day nectar-fed bees (p=0.02, n=27, χ^2=8.5, df=2). No significant relationship was found between the other groups. Two data points were removed, which were identified as outliers based on the results of Grubbs tests (p<0.01, p<0.05). The first outlier had a total of 104 pollen grains and a supercooling point of -7.01°C (n=29, Z=2.89, p<0.05) and the second had a total of 18 pollen grains and had a supercooling point of -7.99°C (n=28, Z=2.88, p<0.05). The frequency of 'high' SCPs was clearly greater in controls and 3 day nectar-only fed samples, compared to 14 day nectar-only fed bees (Figure 2.7), with all 14 day nectar-fed bees having SCPs below -12.42°C.

Neither controls nor 3 day bees were in this lowest SCP group. Additionally, 100% of 14 day nectar fed bees had no pollen in their guts (Table 2.2), compared to 57 and 25% for 3 and 7 days of nectar feeding respectively.

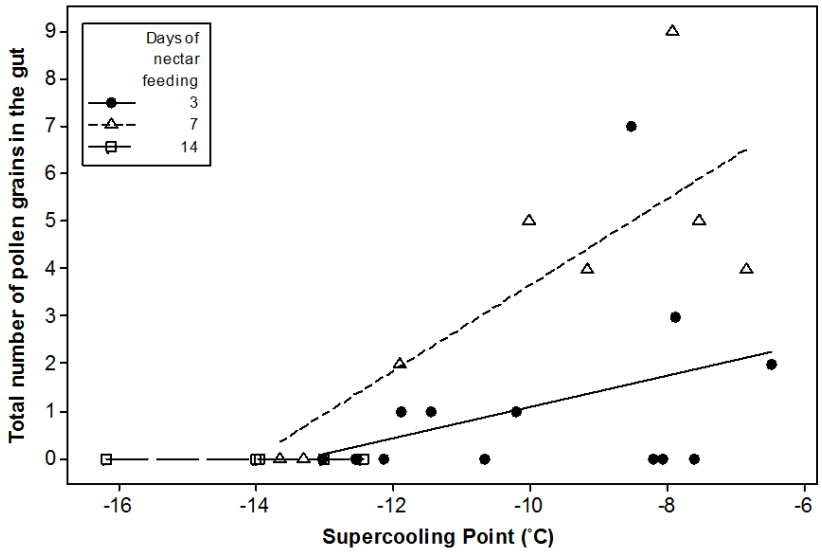

Figure 2.6 The effect of dietary pollen on supercooling point. Total number of pollen grains found in the guts of *Bombus terrestris audax* workers, plotted against their corresponding supercooling points (*n*=14, 8 and 5 for 3, 7 and 14 day nectar-fed workers respectively). For 3 days of nectar feeding, S=1.87, R^2=14.5% R^2(adj)=7.4% p<0.1 y=4.42+0.33x. For 7 days of nectar feeding, S=1.90, R^2=65% R^2(adj)=59.2% p=0.02 y=12.73+0.91x. For 14 days of nectar feeding, no pollen grains were found in the guts of bumblebees.

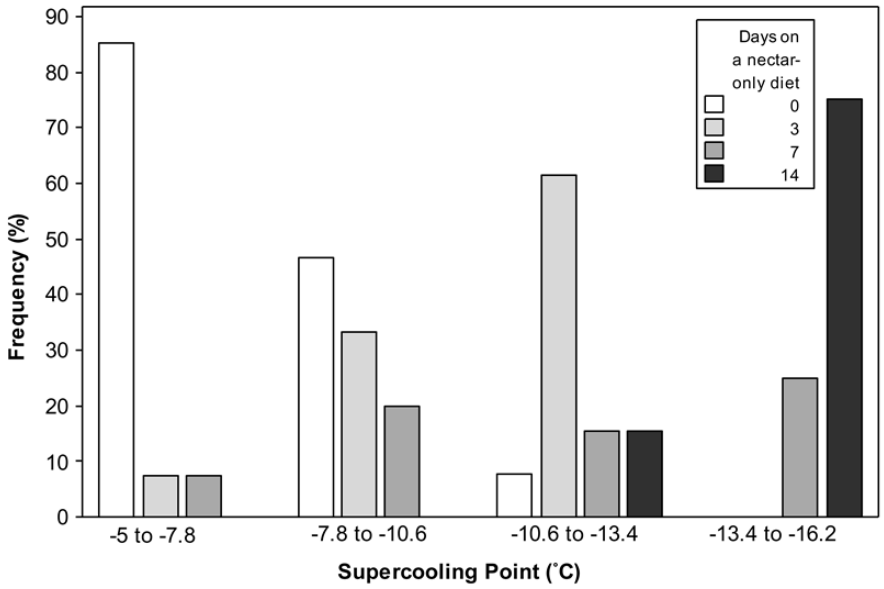

Figure 2.7 Frequency of bumblebee SCPs, partitioned into 4 equal groups, and their corresponding feeding regime. Frequency distributions of supercooling points from *Bombus terrestris audax* workers on a nectar-only diet for 3, 7 and 14 days and controls, *n*=30, 14, 8 and 5 for controls, 3, 7 and 14 days of nectar feeding respectively.

Table 2.2 Proportions of *Bombus terrestris audax* workers with no pollen grains in their guts after 3, 7 and 14 days of nectar-only feeding, *n*=14, 8 and 5 for 3.7 and 14 day nectar-fed workers respectively.

Days of nectar feeding	Percentage of bees with no pollen grains
3	57
7	25
14	100

2.5. DISCUSSION

This study has identified that *B. t. audax* is a freeze avoiding species (see Bale, 1996), with limited supercooling ability (mean SCP of workers -7.1 ± 0.2 and queens -7.4 ± 0.3°C). This is in contrast to several other bee species, *e.g. Osmia cornuta* and *O. rufa*, both with SCPs typically below -24°C (Krunić and Stanisavljevic, 2007) but is comparable with *Megachile rotundata* with SCPs of -8°C (Sheffield, 2008). Despite this limited supercooling ability, both *B. t. audax* workers and queens are able to tolerate exposures to temperatures close to their SCP for short periods; for example, 80 ± 16.3% survived after exposure to -5°C for 2h (Figure 2.3). When subjected to acute (15 min) sub-zero cold stress, 90% of workers were able to survive at -5.0 ± 1.1°C and 50% at -7.8 ± 1.1°C. Indeed, tolerance of sub-zero conditions is actually greater than this result might suggest, because the cooling rate of 0.2°Cmin^{-1} means bees were exposed to temperatures below 0°C for extended periods *e.g.* 65min below 0°C for a -5°C exposure. However, these temperatures represent thermal stresses that can occur across much of the native Northern European range of *B. t. audax* (Cattiaux *et al.*, 2010) during winter, so remaining active could have a significant impact on survival and thus the distribution and abundance of this species. Such is the case for *Nezara viridula* in Japan, whose northern expansion is limited by low January temperatures (Musolin and Numata, 2003). In this regard, it is important to note that it is higher autumn temperatures that most likely influence the physiological 'decision' for insects to avert diapause (Bale and Hayward, 2010), and so become winter active. However, this does not preclude warmer winter conditions. Indeed, climate change is predicted to cause a greater frequency of extreme cold events (Williams *et al.*, 2015).

Bees can of course behaviourally avoid the most extreme conditions by remaining within the subterranean colony. However, if a colony is to survive an entire winter period, workers cannot simply remain within the nest. At some point they must forage for nectar and pollen (potential sources described by Stelzer *et al*., 2010) to fuel colony development and the eventual establishment of the next generation of queens and males.

The ability of insects to rapidly cold harden allows them to mitigate the effect of sudden changes in temperature, *e.g*. during foraging, and fine tune their response to environmental temperature fluctuations (Shreve *et al*., 2004). *Bombus terrestris audax* clearly demonstrates an ability to undergo RCH (Figure 2.4) and, to our knowledge, this represents the first evidence of RCH in Hymenoptera. Interestingly, RCH was not found to lower the SCP of bumblebees, in common with other insects including the Antarctic midge *Eretmoptera murphyi* Schaeffer, 1914 (Diptera: Chironomidae) (Everatt *et al*., 2012), and house fly *Musca domestica* (Coulson and Bale, 1990). RCH is especially relevant to winter activity because bees may need to forage more widely at a time of year when nectar and pollen resources are more limited. Equally, at certain threshold temperatures, activity will become restricted (Chapter 4), meaning that individual bees may not always be able to return to the colony within the same 24 hour period, and thus be exposed to night time temperatures. Given that bumblebees are known to remain away from the colony at night (Free, 1955; Spaethe and Weidenmüller, 2002) and night time winter temperatures in recent years in the UK have reached well below -10°C (Met Office, 2010) this may represent a significant survival risk to *B. t audax*.

In addition to acute cold exposure during foraging, winter active bumblebees are also likely to experience chronic low colony temperatures. While bumblebees do possess the ability to thermoregulate their colonies (Vogt, 1986a; 1986b), a lack of winter floral resources or an excessive thermoregulatory demand, may mean colonies are unable to maintain a favourable temperature (Moret and Schmid-Hempel, 2000). *Bombus terrestris audax* typically constructs nests underground at a range of depths (Alford, 1975), and although this might buffer them from extreme air temperature fluctuations, there is still a survival risk. Recent (2009–2013) winter soil temperatures (10 cm depth) in the UK have consistently fallen below 5°C for many weeks, and below 0°C for periods of several days as far south as Rothamsted, Hertfordshire (UK Environmental Change Network, 2013). Commercially-produced colonies established outside and exposed to winter conditions in Birmingham (2012–13) did not survive to produce any new queens or males, and had very low levels of activity beyond November (Chapter 5). Thus, winter activity caused by warmer autumn conditions disrupting diapause, could have devastating effects on the number of new queens establishing colonies in spring, with associated impacts on pollination service provision. This is not just relevant to *B. terrestris*; other bumblebee species may have their diapause disrupted by climate change as they often have nests close to the surface (Sladen, 1912) or even at elevated sites, *e.g*. in bird boxes (Osborne *et al*., 2008). Here, they may be exposed to temperatures below -5°C (Chapter 5), as well as freeze-thaw transitions.

The $LTime_{50}$ for exposure to 0°C was 7.2 ± 1.1 and 25.6 ± 2.4days for workers and queens respectively. This suggests that active queens have a greater underlying cold tolerance than

workers, even though the SCP of queens and workers were not significantly different. One explanation for this might be the more extensive fat reserves present in diapausing queens to facilitate winter survival and early spring foraging (Goulson, 2010), and increased fat content is often correlated with enhanced cold tolerance (Hahn and Denlinger, 2007). However, it is not known if the fat quantity of active queens differs significantly from workers.

As hypothesised, manipulation of diet through the removal of pollen significantly decreased the SCPs of both workers and queens (Figure 2.5) with every 'pollen free' treatment having a significantly lower SCP than its 'pollen-fed' counterpart. This provides strong support for the ice-nucleating properties of pollen, in agreement with work on *Megachile rotundata* (Krunić, 1971), which showed increased freezing temperatures as a result of dietary ice nucleators. Diet has long been known to affect cold tolerance with Antarctic micro-arthropods shifting their SCPs from a summer 'high' group (mean SCP -7°C) to a winter 'low' group (mean SCP -25°C) after a period of starvation (Worland and Convey, 2001) and freeze avoiding insects evacuating their guts to exclude ice nucleating agents (INAs) (Duman and Patterson, 1978). To sustain winter active colonies, bumblebee workers are required to forage for pollen at low temperatures, therefore behavioural avoidance of ice nucleators is not possible. As a result, workers are at increased risk of freezing mortality when foraging which may lead to poor colony nutrition and death. This poses an unknown risk to winter active colonies at temperatures below -5°C, the highest SCP recorded in this study.

Recent harsh winters (Chapter 5) and an increasing frequency of extreme events (Rosenzweig *et al*., 2001) pose significant threats to existing winter-active colonies. Coupled with a decrease in cold tolerance as a result of ice nucleation, foraging bumblebees are at risk of winter mortality which may lead to colony collapse. This is perhaps why sightings in winter are restricted to the southern UK (Stelzer *et al*., 2010). However, higher autumn temperatures, as a result of climate change, may facilitate winter-active bumblebee colonies in the future. This must be coupled with adequate floral resources, suitable nest sites and the successful production of new queens to establish colonies the following year.

The changing winter phenology of *B. t. audax* is typical of the current climate-change induced threats to pollinators, responsible for disrupting ecosystems and plant-pollinator interactions. In order to understand this trend, future work must include quantification of these changes in a field setting, taking into account activity and pollination behaviour, which is key to understanding the impact of climate change on pollinators.

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CHAPTER 3

CAN NON-NATIVE BUMBLEBEES SURVIVE UK WINTERS? ESTABLISHMENT RISK OF THE COMMERCIALLY-IMPORTED BUMBLEBEE *BOMBUS TERRESTRIS*

3.1. ABSTRACT

Bumblebees have been exported in large quantities to countries outside their native range for the purposes of commercial pollination. In some European countries, the import and release of non-native invertebrate species and subspecies for pollination or biological control is subject to regulation based on national legislation. Where establishment is considered to be undesirable, the same risk assessment methods used to screen non-native invertebrate species can be applied to bumblebees. This study has characterised the cold tolerance of an important non-native commercial pollinator used in the UK, *Bombus terrestris dalmatinus* to inform the likelihood of winter survival and establishment of this subspecies. Lethal temperatures (LTemp), lethal times at 0°C (LTime) and the ability to rapidly cold harden (RCH) were investigated. $LTemp_{10,\ 50}$ and $_{90}$ of workers were -4.6 ± 1.0, -6.1 ± 1.0 and -7.3 ± 3.7˚C respectively, and $LTime_{10,\ 50}$ and $Temp_{90}$ at 0°C were 2.5 ± 1.1, 4.8 ± 1.1 and 9.2 ± 1.1 days respectively. Bumblebees showed a strong RCH response, with survival at -5°C for 8h significantly enhanced by 1h at 0˚C and gradual cooling at 0.2°Cmin^{-1} compared with direct transfer (p<0.01). Worker supercooling points were -7.1 ± 0.2˚C which decreased to -8.4 ± 0.2˚C after a short acclimation period at 0˚C. Non-native bumblebees appear physiologically able to survive moderate UK winters, with a similar cold tolerance profile to the related UK

subspecies, *B. t. audax*. Results are discussed in light of subspecies competition, establishment and commercial pollination.

3.2. INTRODUCTION

Given the economic importance of insect pollination (Gallai *et al*., 2009) and the decline of the north American honeybee, *Apis melifera* (Tentcheva *et al*., 2004), alternative pollinators have increasingly been used to increase the yield and quality of commercially valuable crops. In the past 50 years, demand for commercial pollination has increased by 300%, yet there has only been a 45% increase in managed honey bee hives (Aizen and Harder 2009). Due to improvements in mass rearing methods, bumblebees have been employed as commercial pollinators since 1988 (Inari *et al*., 2005) and are now the sole pollinators of crops such as tomatoes. In 2004, 40,000ha of greenhouse tomatoes (*Lycopersicon esculentum*) worldwide were pollinated by bumblebees, with an estimated value of €12,000 million (Velthuis and van Doorn, 2006). Bumblebees are also deployed in the pollination of alfalfa (*Medicago sativa*), clovers (*Trifolium spp*.), oilseed rape (*Brassica napus*), brown mustard (*Brassica juncea*), sunflower (*Helianthus annuus*) and fruits such as strawberry (*Fragaria x ananassa*), melon (*Cucumis melo*) and kiwifruit (*Actinidia deliciosa*) (Goulson, 2010). Key to their success is their ability to 'buzz pollinate' (Buchmann, 1983) a process which enhances the efficiency of pollen harvesting 400 fold (Winter *et al*., 2006).

The primary bumblebee used in commercial colonies, *Bombus terrestris*, comprises nine

subspecies, each occupying a distinct native range in Eurasia (Rasmont *et al*., 2008). The most widely used subspecies is *Bombus terrestris dalmatinus* (Ings *et al*., 2005a), which is native to South East France, Northern Italy, the Balkanic Peninsulas, Turkey and North Iran (Rasmont *et al*., 2008). Due to the large size of workers and high success rate of colonies, *B. t. dalmatinus* is an attractive pollinator for use in a commercial setting (Velthuis and van Doorn, 2006), and this subspecies has been transported to 57 countries, 16 of which were outside its native range (Ings, 2006). Approximately one million colonies were used worldwide in 2006; however, precautions were not undertaken to identify the threat of establishment in non-native countries (Velthuis and van Doorn, 2006), which led to establishment in Japan, Chile, Argentina, Israel, New Zealand and Tasmania (McFadyen and Lloyd, 2006; Goka, 2010). There was also concern regarding potential competitive displacement of native bee species, heightened in areas of sparse floral resources (Goulson, 2003; Ings *et al*., 2005b). Commercial colonies of *B. t. dalmatinus* are regularly imported into the UK, despite having a native population of *B. t. audax*. Control measures to prevent establishment have been recently introduced in the UK, with 'queen excluders' (devices which prevent queens from exiting colonies) mandatory since January 2013 (Natural England, 2013). However, given the scale of importation to the UK (10,000 colonies annually), it is possible that *B. t. dalmatinus* has already established (Ings *et al*., 2006). When comparing these two subspecies, Ings *et al*. (2006) found consistently higher nectar foraging rates and an increased production of queens in *B. t. dalmatinus*. Consequently, there is a real risk that *B. t dalmatinus* could out-compete the native *B. t. audax*.

In its native Mediterranean environment, *B. t. dalmatinus* typically remains active throughout the year, except for a period of aestivation. This is in contrast to *B. t. audax*, which typically undergoes just one colony cycle per year, with only queens surviving winter in a dormant state termed diapause (Rasmont *et al*., 2008). An important barrier to the establishment of non-native species in regions with a seasonal climate is winter cold. However, the cold tolerance of *B. t. dalmatinus* has never been investigated. This is especially relevant given recent extreme winter events, for example, could *B. t. dalmatinus* have survived recent winters in the UK? Could it become established further north and displace the native *B. t. audax*?

There exists an extensive literature on insect cold tolerance and its application in assessing the establishment risk of introduced species, *e.g*. those employed as biological control agents in glasshouses (Hatherly *et al*., 2005, Hughes and Bale, 2009, Hughes *et al*., 2010, Coombs and Bale, 2014). Ecologically relevant indices of cold tolerance are used to inform the likelihood of establishment of an insect, including the use of LTime and LTemp experiments, which assess survival after exposure to acute and chronic low temperatures. These indices can be used to determine the likelihood of establishment of the non-native bumblebee *B. t. dalmatinus* in the UK. The capacity of winter active workers to respond rapidly to environmental variability is important for winter survival. This can be assessed by measuring rapid cold hardening, a process in which individuals experience increased survival at low sub-zero temperatures after a short period of acclimation (Lee *et al*., 1987). The presence of an RCH response may enable *B. t. dalmatinus* to mitigate the effect of sudden temperature

changes in UK winters and further promote survival. In this study, we employed these ecophysiological indices to compare the cold tolerance of *B. t. dalmatinus* with *B. t. audax*, and determine the likelihood of establishment of *B. t. dalmatinus* in the UK.

3.3. METHODS

Culture system

Mature colonies of *B. t. dalmatinus*, were obtained from Biobest NV (Westerlo, Belgium), maintained at 20ºC in constant darkness and manipulated under red illumination to minimize disturbance (Sadd, 2011). Nectar was available within the colony using a wick system connected to a reservoir of BioGluc® nectar and pollen paste was available *ad libitum* (Biobest NV). For each experimental treatment, 6 replicates of 5 worker bees were used, unless otherwise stated. Control samples of 6 replicates of 5 bees were exposed to 15˚C for the maximum experimental duration and survival was assessed after 72h.

Lethal temperature

Bumblebees were placed into 6 test tubes (n=5 per tube) and secured with a foam bung. Bumblebee thoracic temperature was measured using Type K (exposed wire) thermocouples, connected to a thermocouple data logger (Pico® TC-08 Thermocouple Data Logger). Test tubes were placed into a programmable alcohol bath (Haake Phoenix 11 P2, Thermo Electron Corporation), and were cooled from 20°C, at a rate of 0.2°Cmin^{-1} to a range of sub-zero

temperatures -5, -5.5, -6, -6.5, -7, -8°C). Bumblebees were held at each temperature for 15min before the temperature was increased back to 20°C at 0.2°Cmin^{-1}. Preliminary experiments confirmed that all bees had equilibriated to the holding temperature within 15min. Bees were then transferred to rearing temperature and were housed in 'recovery boxes' in their experimental replicates (6 boxes, with 5 bees per box). Pollen and nectar was available *ad libitum* and survival was assessed after 72 h.

Lethal time at 0°C

Bumblebee workers were placed in groups of 5 to 6 conical flasks (25ml Pyrex). Flasks were held at 10°C in a climate-controlled room for 1 hour to minimise cold shock prior to the experiment. Flasks were then transferred to a Fryka® incubator set at 0°C. A TinyTag® datalogger was added to the incubator to monitor temperature throughout the duration of the experiment. Preliminary experiments confirmed that all bees had equilibriated to the holding temperature within 15min. Bees were maintained in the incubator for a range of durations (2, 4, 5, 7, 9 and 11 days) and were then transferred to a 10°C climate-controlled room for 1 hour to minimise cold shock after the experiment. Bees were then returned to their rearing temperature and were housed in 'recovery boxes' in their experimental replicates (6 boxes, with 5 bees per box). Pollen and nectar was available *ad libitum* and survival was assessed after 72 h. Survival was assessed as previously described.

Rapid Cold Hardening

Determination of the discriminating treatment. These experiments were undertaken at -5˚C, as this was the lowest sub-zero temperature which induced mortality, whilst having no incidence of freezing (SCPs ranged from -5.1 to -10.1˚C). Thirty bumblebees (6 replicates of *n*=5 bees per temperature) were taken from their rearing temperature (20ºC), added to test tubes with thermocouples as previously described, and placed directly in an alcohol bath (Haake® Phoenix 11 P2, Thermo Electron Corporation) set at -5 ºC for a range of durations (2, 4, 6, 8 and 10h). Bees were then re-warmed to rearing temperature at a rate of 0.2ºCmin^{-1} and survival was assessed after 72h. The shortest time duration that resulted in between 10-20% survival was selected as the discriminating treatment (Lee *et al*., 1987).

*Rapid cold hardening response***.** Thirty bumblebees (6 replicates of *n*=5 bees per treatment) were added to test tubes as previously described and exposed to one of two rapid cold hardening regimes prior to transfer to the discriminating treatment: 1h at 0ºC, or gradual cooling at 0.2˚Cmin^{-1} to -5°C. Re-warming post treatment in both experiments was at 0.2°Cmin^{-1} to 20°C, and survival assessed after 72h. Evidence of RCH was determined by any increase in survival relative to direct transfer to the discriminating treatment.

Impact of RCH on supercooling point (SCP)

After a period of 1h at 0˚C, the SCP of 30 workers were measured, using established methods (see Hughes and Bale 2009). Briefly, bees were inserted individually into test tubes containing type K exposed wire thermocouples, placed in an alcohol bath (Haake® Phoenix 11 P2, Thermo Electron Corporation) programmed to cool from 20ºC to -20ºC at a rate of $0.2^{o}Cmin^{-1}$ and freezing exotherms were detected via a computerised recording system.

Statistical analysis

A number of preliminary statistical tests were undertaken on the response variables to determine whether parametric or non-parametric testing could be employed. Normality was tested using Kolmogorov-Smirnov tests; equality of variance (homoscedasticity) was investigated using Levene's tests and time series were plotted to confirm the independence of samples. Pearson's Goodness of Fit tests were also undertaken on LTemp and LTime data to confirm the Probit models fitted the data. LTemp and LTime data were normally distributed and were subsequently analysed via Probit analysis in Minitab® (Finney, 1971). This was used to identify the temperature or time at which 10, 50 or 90% mortality occurred. All RCH data were not normally distributed, but were homoscedastic and independent, therefore independent samples Kruskal Wallis tests with pairwise comparisons were undertaken using SPSS®. As multiple comparison tests in SPSS® include a correction for the familywise error rate, confidence interval bars on multiple comparison bar charts were adjusted using Bonferroni corrections (Figures 3.3 and 3.4). RCH and SCP results were normally distributed, homoscedastic and independent and were analysed using a one way ANOVA in Minitab®.

3.4. RESULTS

Lethal temperature

Worker survival declined rapidly between -5 and -8°C, with 96.6 ± 3.3% survival at -5°C, decreasing to 6.7 ± 4.2% survival at -8°C (Figure 3.1). Using Probit analysis, the temperatures at which bumblebee workers incurred 10, 50 and 90% mortality ($LTemp_{10, 50}$ and $_{90}$) were -4.6 ± 1.0, -6.1 ± 1.0 and -7.3 ± 3.7˚C respectively (regression $p<0.001$, Pearson's Goodness-of-fit $\chi^2=15.4572$, $df=4$ $p=0.004$). There was no mortality in the control sample.

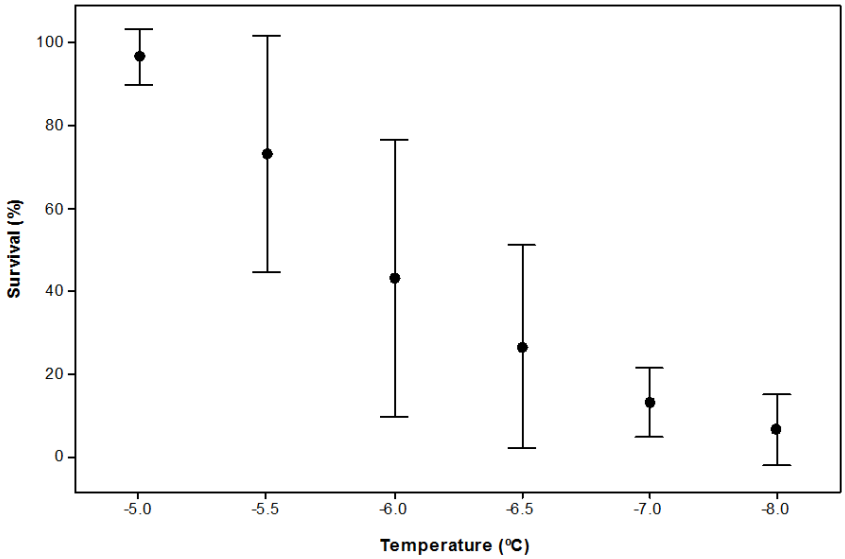


Figure 3.1 Survival of worker bumblebees (*B. t. dalmatinus*) at a range of sub-zero temperatures, each point represents 6 replicates of 5 bees (±2SE).

Lethal time

Worker survival declined particularly rapidly between exposures of 2 and 5 days at 0°C, decreasing from 96.7 ± 3.3% survival to 36.7 ± 20.3%, with a high degree of variability in the 5 day sample (Figure 3.2). After 11 days, survival was 0%. Using Probit analysis, the

durations at 0˚C at which bumblebee workers incurred 10, 50 and 90% mortality ($LTime_{10, 50,90)}$ were 2.5 ± 1.1, 4.8 ± 1.1 and 9.2 ± 1.1 days respectively (regression $p<0.001$, Pearsons Goodness-of-fit $\chi^2=2.6$, $df=4$ $p=0.62$). There was no mortality in the control sample. Worker survival is displayed in Figure 3.2.

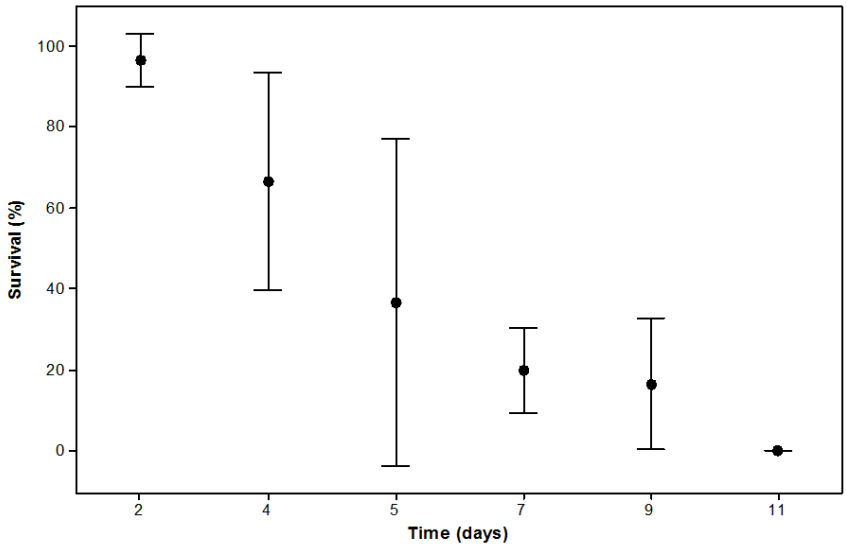


Figure 3.2 Survival of worker bumblebees (*B. t. dalmatinus*) at a range of durations at 0°C. Each point represents 6 replicates of 5 bees (±2SE).

Rapid Cold Hardening

Mean survival of workers at -5˚C decreased with increasing duration of cold exposure, from 96.7 ± 3.3% after 2h exposure, to 6.7 ± 4.2% after 10h exposure (Figure 3.3); 8h at -5°C was chosen as the discriminating treatment (13.3 ± 4.2%). An independent samples Kruskal Wallis test ($H=20.7$, $df=4$, $p<0.01$) with pairwise comparisons indicated survival at 2 and 10h, 4 and 10h and 2 and 8h were significantly different ($p<0.01$, $p<0.05$ and $p<0.05$ respectively).

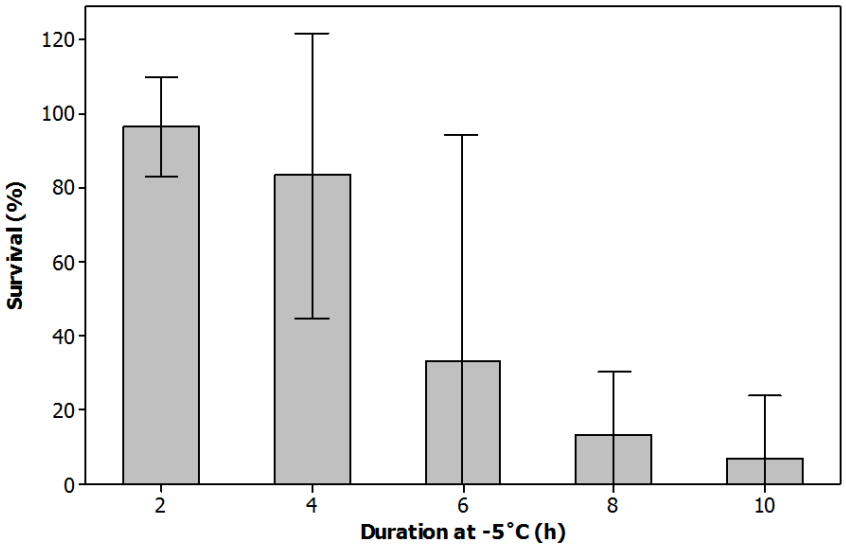


Figure 3.3 Determination of a discriminating treatment for rapid cold hardening in worker bumblebees (*Bombus terrestris dalmatinus*). Mean survival (with Bonferroni-corrected 95% confidence interval bars) of worker bumblebees exposed to periods of 2, 4, 6, 8 and 10h at -5˚C, *n*=30 per temperature.

Survival of workers gradually cooled at 0.2°Cmin^{-1} to the discriminating treatment, or exposed to a pre-treatment of 1h at 0˚C was 100 ± 0% (Figure 3.4). This was significantly higher than survival as a result of direct transfer (H=22.6, df=3, p<0.01).

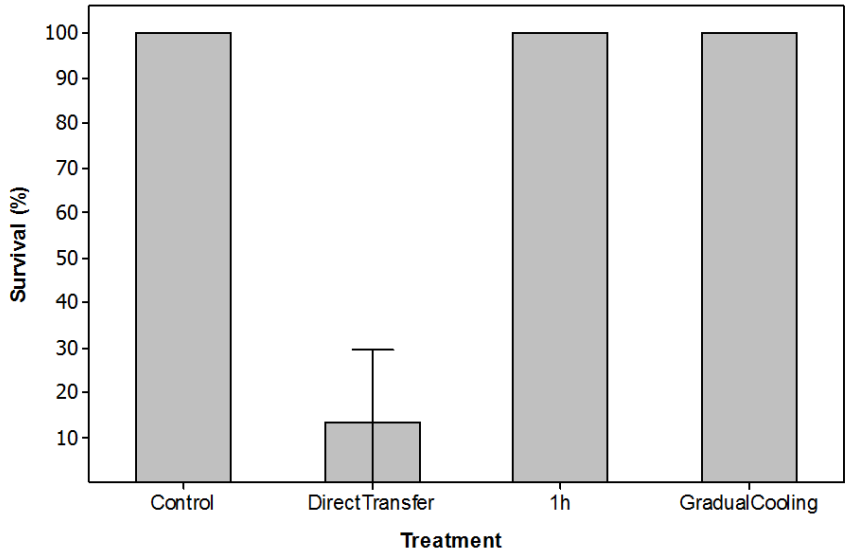


Figure 3.4 A rapid cold hardening response in worker bumblebees (*Bombus terrestris dalmatinus*). Mean survival (with Bonferroni-corrected 95% confidence interval bars) of worker bumblebees after direct transfer to the discriminating treatment (-5°C for 8h), a pretreatment of 1h at 0°C before transfer to the discriminating treatment or gradual cooling (at 0.2°Cmin^{-1}) to the discriminating treatment. *n*=30 per treatment.

A period of 1h at 0°C was sufficient to decrease the SCP of workers, compared to controls (Table 3.1). This suggests RCH has the capacity to lower the SCP of workers and provides a mechanism for the increase in survival after a RCH pre-treatment at 0°C.

Table 3.1 Impact of rapid cold hardening on supercooling point; the supercooling points of workers exposed to a pre-treatment of 1h at 0˚C versus controls. Significant differences are highlighted with an asterisk (*) (*F*=12.4, *df*=59 *p*<0.01).

Treatment group	N	Mean ± SE (°C)	Range (°C)
SCP control	30	-7.1 ± 0.2*	-5.1 to -10.1
SCP following RCH	30	-8.4 ± 0.2*	-5.6 to -12.2

3.5. DISCUSSION

Characterising the ability of non-native terrestrial invertebrates to survive winter is critical to assessing establishment risk in the UK (Bale and Hayward, 2010). There is a considerable body of work addressing this issue for glasshouse biocontrol agents (Hatherly *et al*., 2005, Hughes and Bale, 2009, Hughes *et al*., 2010, Coombs and Bale, 2014), but far less attention has been paid to commercial pollinator species. In 1989, when *B. t. dalmatinus* were first used in imported commercial colonies (Kwon, 2008), there was little restriction on the use the non-native bumblebees, *B. t. dalmatinus*, for the purposes of pollination (Ings *et al*., 2005b). This led to concerns regarding the establishment of the subspecies (Ings *et al*., 2006). Restrictions were introduced in 2013, with *B. t. dalmatinus* licensed only for pollination in glasshouses and polytunnels in the UK with 'queen excluders' (Natural England, 2013). However, many believe these restrictions were implemented too late to prevent establishment, and *B. t. dalmatinus* may be established in the UK (Ings *et al*., 2006). Given this, and the fact that this subspecies is active year round, it is pertinent to assess the ability of workers and winter active colonies to survive UK winter conditions. This will also provide insight regarding the risk of *B. t. dalmatinus* displacing native *B. t. audax*.

The current study has identified *B. t. dalmatinus* as a freeze avoiding species (see Bale 1996), with limited supercooling ability (mean SCP of workers was -7.1 ± 0.2). This is comparable to *Megachile rotundata* (-8°C; Sheffield 2008) and *Bombus terrestris audax* -7.1 ± 0.2°C (Chapter 2). Despite this limited supercooling ability, workers were able to tolerate exposures

to temperatures close to their SCP for short periods; for example, 97 ± 3.3% survived exposure to -5˚C for 2h (Figure 3.3). Workers experienced 10% mortality at -4.6 ± 1.0˚C, and 50% mortality at -6.1 ± 1.0˚C. This suggests that the SCP of the subspecies represents the lower limit to survival, with little mortality attributed to cold shock. Bumblebees are therefore likely to survive sub-zero temperatures if freezing does not occur. Similar indices have been investigated in UK-native bumblebees, *B. t. audax* (Chapter 2) and comparable $LTemp_{10}$ and $LTemp_{50}$ values were -5.0 ± 1.1 and -7.8 ± 1.1˚C respectively. This suggests a high degree of similarity (given their non-overlapping fiducial limits) between the cold tolerance profile of the two subspecies, despite their geographical separation. Given the similarity between the cold tolerance profile of *B. t. dalmatinus* and the native *B. t. audax*, evidence presented in the current study suggests there is significant risk of establishment of *B. t. dalmatinus* in the UK.

The ability of workers to survive UK winter temperatures is essential to determining the ability of the subspecies to establish in this country. Daytime temperatures in winter are often below 0°C (Met Office, 2013) and have the potential to cause mortality in active, worker bumblebees with SCPs and LTemps given the results of the present study. For example, in February 2012, a maximum daytime winter temperature of -5°C was recorded in Lincolnshire (Met Office 2012), which overlaps SCP results. Temperatures below -5.3°C are likely to cause some freezing mortality in *B. t. dalmatinus*, and would impact the ability of workers to return forage to a colony. As colonies rely on workers to bring high levels of forage back to the colony to maintain an optimum brood temperature (Heinrich, 1975), a lack of forage would be predicted to impact colony longevity and growth (Weidenmüller *et al*., 2002). If

foraging workers were unable to return to the colony at night, they would be exposed to sub-zero winter temperatures which, for example, were as low as -7.7°C in February 2014 (Met Office, 2014). Low temperatures of this nature have the potential to cause freezing mortality in a significant number of workers, given that a temperature of -6.1 ± 1.0˚C caused 50% mortality when investigated in the current study. Although the lower thermal limits of *B. t. dalmatinus* overlap with winter temperatures in the UK, this is also the case for the native *B. t. audax*, given the degree of similarity between cold tolerance indices (Chapter 2). It follows, therefore, that *B. t. dalmatinus* may be in direct competition for resources with winter-active *B. t. audax* in the south of the UK.

Acute cold temperatures are useful in screening potential invasive species for risk of establishment (Hatherly *et al*., 2005). However, chronic low temperature exposure also provides information about the long term survival of the organism. Bumblebees inhabit underground colonies which can provide refuge from extreme low temperatures in winter (Sladen, 1912). A lack of winter floral resources or an excessive thermoregulatory demand may mean colonies are unable to maintain a favourable temperature (Moret and Schmidt-Hempel, 2000) and may equilibrate to the temperature of the surrounding earth. The ability of *B. t. dalmatinus* to survive chronic exposure to 0°C was examined in the current study. Workers suffered 10% mortality after 2.5 ± 1.1 days and 50% after 4.8 ± 1.1 days, suggesting an inability to survive in unheated, subterranean colonies. Winter active *B. t. dalmatinus*, would therefore have to invest foraging resources in maintaining a high colony temperature, representing a high thermoregulatory demand. These results are in line with the LTime values

recorded for *B. t. audax*, with 10% mortality after 2.3 ± 1.2 days and 50% after 7.2 ± 1.1 days (Chapter 2). Both subspecies are likely to suffer mortality after chronic exposure to temperatures frequently experienced in northern Europe during winter.

The ability of *B. t. dalmatinus* to survive in the UK may be improved if individuals are able to undergo rapid cold hardening. Rapid cold hardening allows insects to mitigate the effect of sudden changes in temperature, for example when foraging, and fine tune their response to environmental temperature fluctuations (Shreve *et al*., 2004). RCH has been exhibited by *B. t. audax* (Chapter 2), a species which occupies colder climates than the Mediterranean *B. t. dalmatinus* (Rasmont *et al*., 2008) and may facilitate survival in winter months. Interestingly, the current study has shown that *B. t. dalmatinus* is also able to undergo RCH (Figure 3.4), despite the fact that it is exclusively found in the Mediterranean region. With a pre-treatment of 1h at 0°C or gradual cooling at 0.2˚Cmin^{-1} survival increased to 100%, compared with 13.3 ± 4.2% for bees transferred directly to the discriminating treatment.

RCH could be critical to the survival of workers under winter conditions in the UK, given that minimum air temperatures have reached -5°C in recent winters (Chapter 5), and without RCH, survival declines rapidly at this temperature (Figure 3.3). The fastest rate of temperature decline recorded in air temperature during the winter of 2011 was 0.03°Cmin^{-1}, suggesting RCH could be induced under field conditions (Chapter 4). Interestingly, RCH was also found to lower the mean SCP of *B. t. dalmatinus* (Table 3.1), which is in contrast with

several other insect species where SCP was not affected by RCH; for example, the Antarctic midge, *Eretmoptera murphyi* Schaeffer (Diptera: Chironomidae) (Everatt *et al*., 2012), and house fly, *Musca domestica* (Coulson and Bale 1990). The decrease in SCP is predicted to be the result of accumulation of substances which decrease SCP such as sodium chloride, urea, glycerol, and glucose (Wilson *et al*., 2003).

The presence of rapid cold hardening in a Mediterranean species is not uncommon; for example RCH has been identified in the olive fruit fly *Bactrocera oleae* Gmelin (Diptera, Tephritidae), in Greece, where survival increased from 5 to 80-92% following a 2h period of acclimation at various temperatures between 0 and 10˚C, before transfer to the discriminating treatment (2h at -6.5˚C) (Koveos, 2001). However, the ability to rapidly cold harden in winter active bees in the UK may facilitate survival and promote foraging and thus colony sustainability at low temperatures. This is likely to prove highly advantageous to bees operating close to their lower physiological limits and may facilitate naturalization of *B. t. dalmatinus* in the UK and its spread northwards.

Several studies have suggested that *B. t. dalmatinus* may have already established in the UK (Ings *et al*., 2005b; 2006). The cold tolerance of this subspecies is certainly similar to that of its UK-native conspecific, and given the larger size and superior foraging performance of *B. t. dalmatinus* workers, it may outcompete *B. t. audax*. Competition for floral resources is known to be heightened in areas of patchy floral resources (Goulson 2003), and given the recent

decline in resource ability (Potts *et al*., 2010a) and phenological mismatching between plants and pollinators as a result of climate change (Schweiger *et al*., 2010), the detrimental effects of competition are predicted to be exacerbated. Ings *et al*. (2005) have also presented the possibility of hybridisation between *B. t. audax* and *B. t. dalmatinus*, with mating occurring between the two subspecies. This scenario may be possible if the two subspecies are spatially and temporally interacting, and may result in genetic homogenisation and the loss of genetic diversity (Ings, 2006).

Climate change may further facilitate the establishment of *B. t. dalmatinus*, given the predicted increase in mean global surface temperature of between 0.3 and 0.7°C (for the period 2016 to 2035) (IPCC, 2014). However, there is evidence that climate change will bring more frequent extreme cold events in winter (Rosenzweig *et al*., 2001). Under these more extreme environment scenarios, it may be *B. t. audax* that has the advantage, as diapausing queens will be better able to survive than winter active colonies. However, during years when winters are mild, *B. t. dalmatinus* workers seem as capable as *B. t. audax* workers to tolerate cold conditions. Therefore, if warmer winters are coupled with adequate floral resources, and suitable nest sites, then *B. t. dalmatinus* may outperform *B. t. audax* in producing new queens to establish colonies the following year. Thus, these data suggest a significant risk to *B. t. audax* as a result of *B. t. dalmatinus'* establishment, resulting in the decline of a commercially valuable, UK-native pollinator. This scenario highlights the importance of conducting environmental risk assessments before releasing non-native insects, even if the subject is considered a beneficial pollinator. Research now needs to move beyond the cold tolerance of

individual workers, and use controlled field trials to determine whether either subspecies can sustain a colony throughout winter.

This chapter has been submitted for publication in Apidologie.

CHAPTER 4

BUMBLEBEE ACTIVITY IN A CHANGING CLIMATE: THERMAL ACTIVITY THRESHOLDS OF NATIVE (*BOMBUS TERRESTRIS AUDAX*) AND NON-NATIVE (*BOMBUS TERRESTRIS DALMATINUS*) BUMBLEBEES

4.1. ABSTRACT

Bumblebees are increasingly exposed to extreme low and high temperatures as a result of winter activity and climate warming respectively. Despite this, their thermal limits are currently unknown. Thermal activity thresholds were investigated in workers of the UK-native *Bombus terrestris audax* and the non-native *Bombus terrestris dalmatinus* subspecies, often used in commercial pollination. CTmin, chill coma, chill coma recovery, activity recovery, CTmax and heat coma were investigated in both subspecies, including the effects of a period of high and low temperature acclimation. Results showed *B. t. audax* had a significantly lower CTmin, chill coma and chill coma recovery temperature than *B. t. dalmatinus* (p<0.01, 0.03 and <0.01 respectively), suggesting the ability for the subspecies to remain active at lower temperatures. Low temperature acclimation (10°C for 7 days significantly lowered the CTmin and chill coma temperature of *B. t. dalmatinus* (p=0.02 and 0.01), while acclimation at 30°C significantly elevated heat coma temperature (p=0.03), suggesting an adaptive advantage of acclimation. *B. t. audax* saw no positive effects of acclimation (p>0.05). These results are discussed in the light of native versus non-native bumblebees and the sustainability of winter active bumblebees in an era of climate change.

4.2. INTRODUCTION

Bumblebees native to northern Europe, such as *Bombus terrestris audax*, are typically univoltine, with queens overwintering in a state of diapause (Sladen, 1912). Activity is usually restricted to the more favourable summer months and colony death typically occurs in mid-Autumn (Alford, 1969). However, numerous anecdotal reports, as well as a recent study by Stelzer *et al*. (2010), have confirmed winter activity in *B. t. audax* in the south of the UK, with foraging workers observed in urban parks and gardens. This winter activity is facilitated by an abundance of winter flowering plants (for examples, see Appendix A) providing a source of pollen and nectar over winter (Stelzer *et al*., 2010). Urban environments can also experience temperature conditions 1-3°C higher than their rural counterparts (Grimmond, 2007). Even taking this into account, the occurrence of winter active colonies raises the problem that workers will be exposed to temperature conditions far below those they typically experience in summer. Equally important is their ability to forage and thus sustain a winter colony. This depends much more on the ability of bees to sustain coordinated movement, and can be determined experimentally by assessing thermal activity thresholds (as in Hazell *et al*., 2008).

Thermal activity thresholds measure the limits to movement at high and low temperatures, and provide information about the thermal limits for key processes such as foraging, reproduction and predator avoidance (Hazell and Bale, 2011). When experimentally decreasing the ambient temperature at a constant rate, an organism will first reach the minimum temperature for coordinated movement termed the critical thermal minimum

(CTmin), associated with the disruption of muscle action potentials (MacMillan and Sinclair, 2011). If the temperature is lowered still, the organism will enter a reversible state of coma, associated with the accumulation of chill injuries (Hazell and Bale, 2011). These are the result of phase changes in membranes and alterations to metabolism (Renault *et al*., 2004). The temperatures at which an insect is able to recover from chill coma and regain coordinated movement are termed chill coma recovery and activity recovery respectively (Hazell and Bale, 2011). These indices have been used particularly in studies of invasive species for ecological niche modelling and predicting spatial range expansion (Hill *et al*., 2013). In this study, I use these indices to assess the potential for bumblebee temporal range expansion into periods of winter activity within the UK.

In studies where ambient temperature is gradually increased, an organism will first experience the maximum temperature for coordinated movement, the critical thermal maximum (CTmax) before entering heat coma (Hazell *et al*., 2008). Due to proximity of heat coma temperatures to those which cause heat mortality, heat coma recovery cannot often be achieved (Alford *et al*., 2012). Understanding the upper limits of coordinated activity will allow an insight into the pollination capacity of *B. terrestris* in the range of non-native countries it is currently exported to (Ings *et al*., 2005b).

Historically, activity thresholds have been investigated in non-native biological control agents and their prey (Hatherly *et al*., 2005) to quantify the efficacy of biological control agents under different temperature conditions. For example, activity thresholds have been

determined in the predatory mirid *Nesidiocoris tenuis* (Reuter) (Hemiptera: Miridae) (Hughes *et al*., 2010), and the parasitic wasp *Lysiphlebus testaceipes* Cresson (Hymenoptera: Aphidiidae) as well as their aphid prey (Hughes *et al*., 2011). Cold acclimation is known to decrease the CTmin and/or chill coma temperature in some species (Hughes *et al*., 2010; Alford *et al*., 2012), and thus provides insects with an opportunity to improve their ability to remain active at low temperatures.

Most insects are entirely dependent on passive environmental heat (poikilothermy) to maintain their body temperature (Bale, 2002) and so climate conditions strongly influence activity. Some species 'bask' to elevate body temperatures above ambient conditions (Hodkinson, 2005), but the capacity to sustain this activity at low temperatures is very limited. For insects able to generate their own heat, however, such as bumblebees, there is significant potential to remain active at low temperatures (Heinrich, 1975). The endothermic capacities of bumblebees are well reported, with queen bumblebees able to forage at temperatures as low as 10˚C (Heinrich, 1979). Stone and Willmer (1989) also found worker bumblebees were able to warm up by 1.6˚C in 10 seconds, up to a stable flight temperature of 33.3 ± 0.3˚C. This heat generation is created by simultaneous contraction of flight muscles in the thorax, as opposed to alternating contraction when flying (Goulson, 2010). This generates an audible buzzing which is correlated with metabolic heat flux (Schultze-Motel and Lamprecht, 1994) and a pumping of the abdomen (Stone and Willmer, 1989). Flight muscle metabolism is thought to be generated by actinomyosin, ATPase and oxidative phosphorylation (Staples *et al*., 2004). This study aims to undertake the first ever activity threshold assessment of an endothermic insect, to determine if *B. t. audax* or *B. t. dalmatinus*

are able to elevate their body temperatures above ambient, and remain active at temperatures below their rearing conditions (20°C). This will provide evidence to determine whether bumblebees can remain active in UK winter conditions, such as those recorded in Chapter 5 (Figure 5.4).

Foraging activity is of course essential to the survival of winter-active colonies, as continuous provisions are required to sustain larvae, nest workers and the egg-laying queen (Weidenmüller *et al*., 2002). With unfed workers surviving only 20-30 hours without food (Moret and Schmidt-Hempel, 2000), even short-term environmental temperature fluctuations below worker activity thresholds have the potential to prevent foraging and thus cause colony death. Assessing thermal activity thresholds of commercial bees is also valuable as it will define scenarios under which bumblebees could be used for pollination services under different climate scenarios and regionally (within UK and EU). For example, could bumblebees be used to pollinate winter grown plants? Additionally, in times of pollinator decline (Potts *et al*. 2010a), commercial bumblebees have been known to compensate for the losses of other pollinators (Heard *et al*., 2007), Thus, low activity thresholds might mean bumblebees could mitigate against climate change where the loss of early spring or late autumn pollinators impacts on pollination service provision.

Equally important are high temperature activity thresholds. Bumblebees such as *B. t. dalmatinus* are commercially produced and exported to many non-native countries such as Asia, South America and Australasia (Ings *et al*., 2005a; Velthuis and van Doorn, 2006).

Given the decreased pollination efficiency observed at high temperatures in *B. terrestris* (Kwon and Saeed, 2003), an investigation of the high temperature thresholds may prove informative. With an optimum foraging temperature of 25.7°C, Kwon and Saeed (2003) recorded high rates of bumblebee pollination in greenhouse hot pepper *Capsicum annuum* L. However, at higher temperatures (32.7°C), foraging activity decreased by 69.7%. The reasons for this declining performance remain untested, but it is important to note that flower temperatures can be well above ambient conditions (personal observations). Thus, bees may be exposed to much higher temperatures than climate data suggest, with associated impacts on activity.

This study aims to elucidate activity thresholds in native and non-native bumblebee workers to inform the capacity of both subspecies to remain active at low UK temperatures. It will also assess upper activity thresholds relevant to flower temperatures recorded in the field. In addition, the capacity of each species to shift their activity windows through acclimation is investigated. This will inform both the likelihood of establishment of *B. t. dalmatinus* in the UK and the possible effects of climate change on both subspecies.

4.3. METHODS

Culture system

Mature colonies of *B. t. audax* and *B. t. dalmatinus* obtained from Biobest NV (Westerlo, Belgium), were maintained at 20ºC in constant darkness and manipulated under red illumination to minimise disturbance (Sadd, 2011). Nectar was available within the colony using a wick system connected to a reservoir of BioGluc nectar and pollen paste was available *ad libitum* (Biobest NV). Control bees were taken from these 20°C cultures, while cold acclimated bees and warm acclimated bees were kept in feeding boxes containing pollen and nectar for 7 days, at 10 or 30ºC respectively.

Experimental System

The thermal activity thresholds of *B. t. audax* and *B. t. dalmatinus*, were assessed in a temperature-controlled arena within an aluminium block (as described by Hazell *et al*., 2008). Arena temperature was controlled via a programmable alcohol bath (Haake Phoenix 11 P2, Thermo Electron Corporation) and recorded using a thermocouple (Tecpel® Advanced Digital Thermometer DTM-315, Heatmiser, UK), which was displayed with the video recording using playback software (Studio Capture DT, Studio86Designs, Lutterworth, UK). Bees were placed in the centre of the arena, in groups of 2, which was then covered by a thin disc of Perspex. Activity was recorded via a digital video camera (Infinity 1-1- Lemenera Scientific, Ottawa, Canada) with a macro lens (Computar MLH-10X, CBC Corp., USA). Videos were played back to determine the temperature at which the last coordinated

movement (CTmin) and the last twitch (chill coma) occurred. If bees entered a position in which a clear view of their appendages was not possible, they were discounted from the analysis, as this prevented CTmin and chill coma being visually determined. Experiments were repeated with a fresh sample of bees each time, until 15 bees of each species were recorded entering CTmin and chill coma.

Monitoring the temperature of the area alone does not provide an accurate measurement of the body temperature of bumblebees within the arena. Consequently, a series of calibration experiments (as in Coombs and Bale 2013; 2014) was undertaken to track bumblebee thoracic temperature at every degree, so that the rate of bee temperature change could be plotted against arena temperature change for both the cooling and warming regimes set out in future sections. Bumblebees were added to the arena in groups of 2 and bumblebee thoracic temperature was measured using Type K (exposed wire) thermocouples which were attached to the thorax of each bee with fine twine. Preliminary experiments confirmed that contact was maintained between the thermocouple and the thorax throughout the experimentation period. The thermocouples were connected to a data logger (Pico® TC-08 Thermocouple Data Logger), interfaced with a computer. A further thermocouple (Tecpel® Advanced Digital Thermometer DTM-315, Heatmiser, UK) simultaneously recorded the temperature of the arena. Bumblebee and arena temperature were recorded every degree from 20 to -10°C (low temperature calibration) and from 20 to 55°C (high temperature calibration). Linear regression analysis was undertaken for each experimental regime (low and high) for each subspecies (*B. t. audax* and *B. t. dalmatinus*) n=10, 10, 8, and 6 for low *B. t. audax*, high *B. t. audax*, low *B. t. dalmatinus* and high *B. t. dalmatinus* respectively. The equations generated were used to adjust the

experimental values recorded in activity threshold experiments (*i.e.* CTmin, chill coma etc.). Controls recorded no mortality as a result of this procedure.

Critical thermal minimum (*CTmin*) *and chill coma*

Bumblebees were allowed to settle in the arena for 30min at the rearing (20°C) or cold acclimation (10°C) temperature. The arena was then cooled to -10 ºC at a rate of 0.1ºCmin^{-1}, a temperature at which 100% of specimens had entered chill coma. Videos were retrospectively analysed and the minimum temperature for coordinated movement (CTmin) and the last movement of an appendage (chill coma) were recorded, following the methods of Hazell *et al*. (2008).

Chill coma recovery and activity recovery

A fresh sample of bumblebees was used and placed in the arena as previously described. The arena temperature was lowered from 20 to -7ºC at 0.1ºCmin^{-1}, held for 15min and re-warmed at the same rate. An arena temperature of -7ºC represented a temperature at which 100% of specimens had entered chill coma in previous experiments. The video recording was retrospectively analysed and the temperatures at which bumblebees first moved an appendage (chill coma recovery) and regained coordinated movement (activity recovery) were recorded.

Critical thermal maximum (*CTmax*) *and heat coma*

Bumblebees were placed in the arena as previously described and temperature was increased from the rearing (20°C) or warm (30°C) acclimation temperature at a rate of 0.1ºCmin^{-1} to 55ºC, a temperature at which 100% of specimens had entered heat coma. Videos were retrospectively analysed and the maximum temperature for coordinated movement (CTmax) and the last movement of an appendage (heat coma) were recorded. As in previous studies, heat coma recovery experiments were not undertaken as entry into heat coma was lethal (Coombs and Bale, 2013; Hazell *et al*., 2008; Hughes *et al*., 2010).

Statistical analysis

A number of preliminary statistical tests were undertaken on the response variables to determine whether parametric or non-parametric testing could be employed. Normality was tested using Kolmogorov-Smirnov tests; equality of variance (homoscedasticity) was investigated using Levene's tests. Time series plots of response variables suggested no linkage or pairing of data, and were used to confirm the independence of samples. Linear regression was undertaken on Minitab® to calibrate the arena temperature and generate fitted line plots. Regression models were checked using graphical analysis of residuals, including their normality, fit and order. Regression equations were subsequently used to adjust experimental values to reflect bumblebee temperature. Data for each activity threshold experiment was normally distributed, homoscedastic and independent, therefore analysed using analysis of variance (ANOVA) tests on SPSS® were used. Tukey's HSD tests were then used to identify significant differences between activity thresholds and acclimation

treatments for both subspecies. Results were considered significant when $p<0.05$. As multiple comparison tests in SPSS® include a correction for the familywise error rate, confidence interval bars on multiple comparison bar charts were adjusted using Bonferroni corrections (Figures 4.6, 4.7 and 4.8).

4.4. RESULTS

Calibrations

The thoracic temperatures of *B. t. audax* and *B. t. dalmatinus* deviated little from those of the arena, suggesting an absence of endothermy (Figures 4.1-4.4). The equations generated from the regression analyses were used to calculate the temperature of the bee (x) when the arena temperature (y) was known. Equations generated are reported in Table 4.1.

Table 4.1 Equations generated, as the result of regression analysis, from low (20 to -10°C) and high (20 to 55°C) calibrations comparing *B. t. audax* and *B. t. dalmatinus* thoracic temperatures with the temperature of an aluminium arena.

***Bombus terrestris* subspecies**	**Temperature regime**	**Equation**	**R^2 (%)**	**p**
audax	Low	y=1.568+0.9282x	98.0	<0.001
audax	High	y=1.230+0.9359x	98.3	<0.001
dalmatinus	Low	y=0.4277+0.9648x	95.0	<0.001
dalmatinus	High	y=4.360+0.8743x	95.8	<0.001

Using the above equations, arena temperature could be used to calculate an estimated value for bumblebee body temperature.

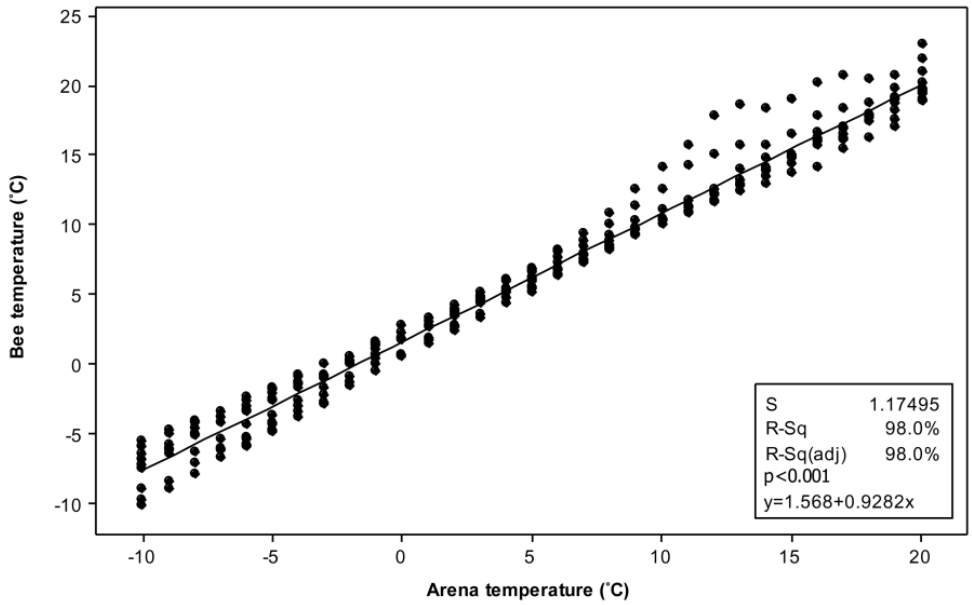


Figure 4.1 Low temperature calibration of *Bombus terrestris audax* individuals (*n*=10). The temperature of bumblebees contained in aluminium activity arena, plotted against the simultaneous temperature of the arena. Temperatures were recorded in 1˚C increments from 20 to -10˚C.

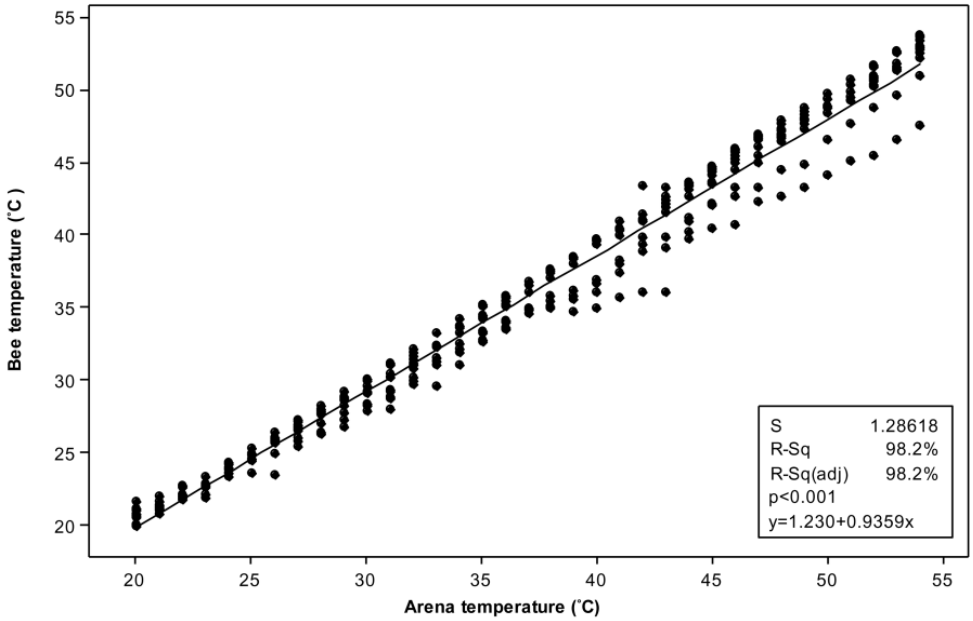


Figure 4.2 High temperature calibration of *Bombus terrestris audax* individuals (*n*=10). The temperature of bumblebees contained in an aluminium arena, plotted against the simultaneous temperature of the arena. Temperature was recorded in 1˚C increments from 20 to 55˚C.

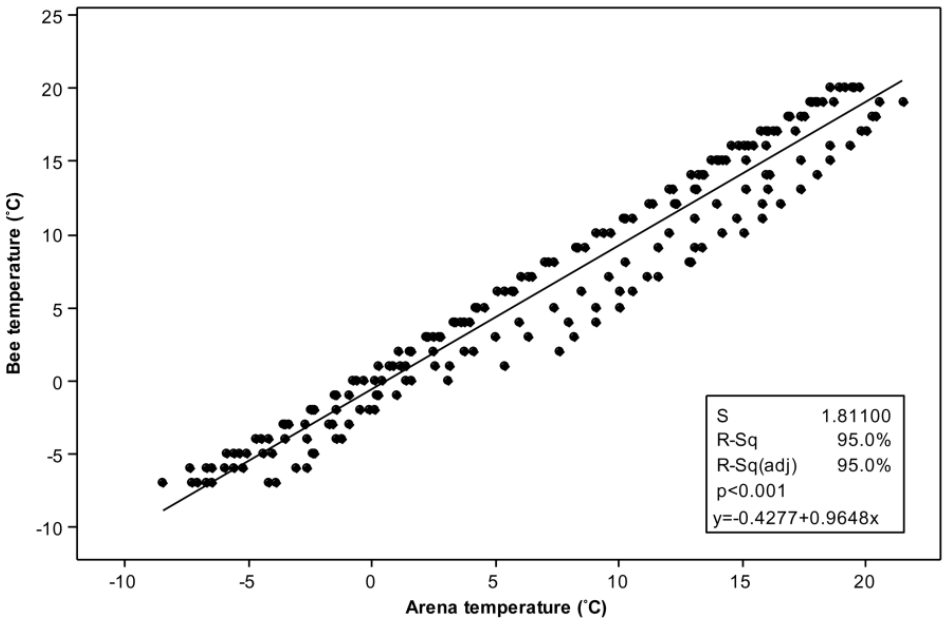


Figure 4.3 Low temperature calibration of *Bombus terrestris dalmatinus* individuals (*n*=8). The temperature of bumblebees contained in an aluminium arena, plotted against the simultaneous temperature of the arena. Temperature was recorded in 1˚C increments from 20 to -7˚C.

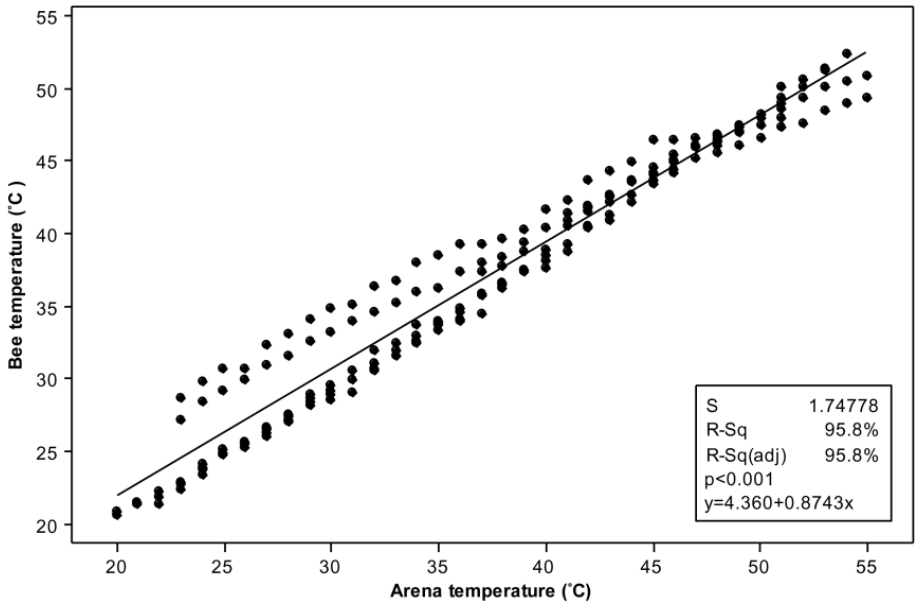


Figure 4.4 High temperature calibration of *Bombus terrestris dalmatinus* individuals (*n*=6). The temperature of bumblebees contained in an aluminium arena, plotted against the simultaneous temperature of the arena. Temperature was recorded in 1˚C increments from 20 to 54˚C.

Assessing endothermy in wild vs. commercial bees

As there was no evidence of endothermy in commercial strains of either *B. t. audax* or *B. t. dalmatinus* (Figures 4.1-4.4), it was hypothesised that either: 1) the physiological response to elevate body temperature above ambient conditions had been lost in commercial cultures, or 2) the activity threshold experimental set-up did not induce an endothermic response. To test this, it was decided to repeat the calibration experiment using wild *B. t. audax* workers collected from Winterbourne Botanical Gardens, Birmingham, UK (52˚27'13"N 1˚55'29"W) between April and May 2014. Individuals were collected and transferred directly to the aluminium arena, where temperature was lowered from 20°C to 0°C at a rate of 0.1˚Cmin^{-1} as described previously. Wild workers were released back to Winterbourne Botanical Gardens after experimentation. Thoracic temperatures of wild bees (Figure 4.5) tracked the arena temperature in much the same way as commercial bees did (Figures 4.1-4.4), although they managed to maintain a slightly higher temperature: for example, when the arena was at 0°C, the average temperature of the wild bees was 2.9 ± 0.4°C, whereas commercial bees recorded a slightly lower temperature of 1.6 ± 0.3°C. It must be noted, however, that wild bee temperatures in this study were only recorded down to a minimum of 0°C, compared to a minimum of -10°C in commercial workers. This was because wild bees were released after experimentation.

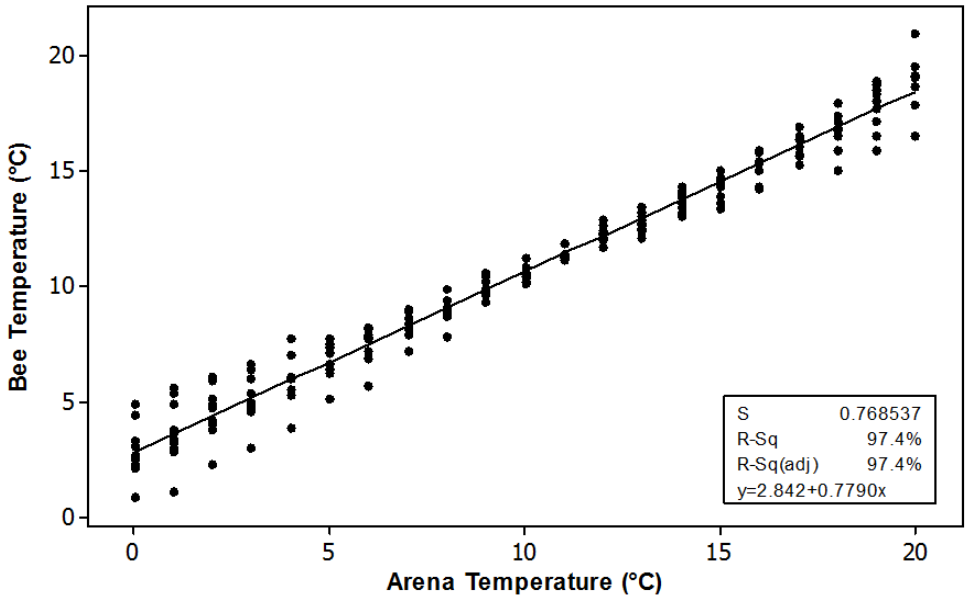


Figure 4.5 Low temperature calibration of wild *Bombus terrestris audax* workers (n=10). The temperature of bumblebees contained in an aluminium arena, plotted against the simultaneous temperature of the arena. Temperature was recorded in 1˚C increments from 20 to 0˚C. The temperature of the arena was not lowered beyond 0°C to ensure bumblebees did not suffer mortality. Bumblebees were subsequently released back to the collection site (Winterbourne Botanical Gardens), p<0.001.

CTmin and chill coma

The mean CTmin temperature for non-acclimated *B. t. audax* was 4.2 ± 0.5˚C, while in bees acclimated at 10°C, this value was 5.8 ± 0.7˚C (Figure 4.6a). The mean (± SE) CTmin temperatures for non-acclimated and acclimated *B. t. dalmatinus* were 9.6 ± 0.5 and 6.9 ± 0.8˚C respectively. Acclimation had a significant lowering effect on the CTmin of *B. t. dalmatinus* but had no effect on *B. t. audax* (p=0.02 and p<0.1 respectively, Tukey's HSD test). When comparing non-acclimated subspecies, *B. t. audax* had a significantly lower CTmin than *B. t. dalmatinus* (p<0.01).

The mean (± SE) chill coma temperature in non-acclimated *B. t. audax* was -3.1 ± 0.3˚C and in acclimated bees, this value was -3.9 ± 0.3˚C (Figure 4.6b). The mean (± SE) chill coma temperature for non-acclimated *B. t. dalmatinus* was -1.8 ± 0.2˚C and in acclimated bees, this value was -3.2 ± 0.5˚C. Acclimation had a significant lowering effect on the chill coma of *B. t. dalmatinus* but had no effect on *B. t. audax* (p=0.01 and p>0.1 respectively, Tukey's HSD test). When comparing non-acclimated subspecies, *B. t. audax* had a significantly lower chill coma than *B. t. dalmatinus* (p=0.03).

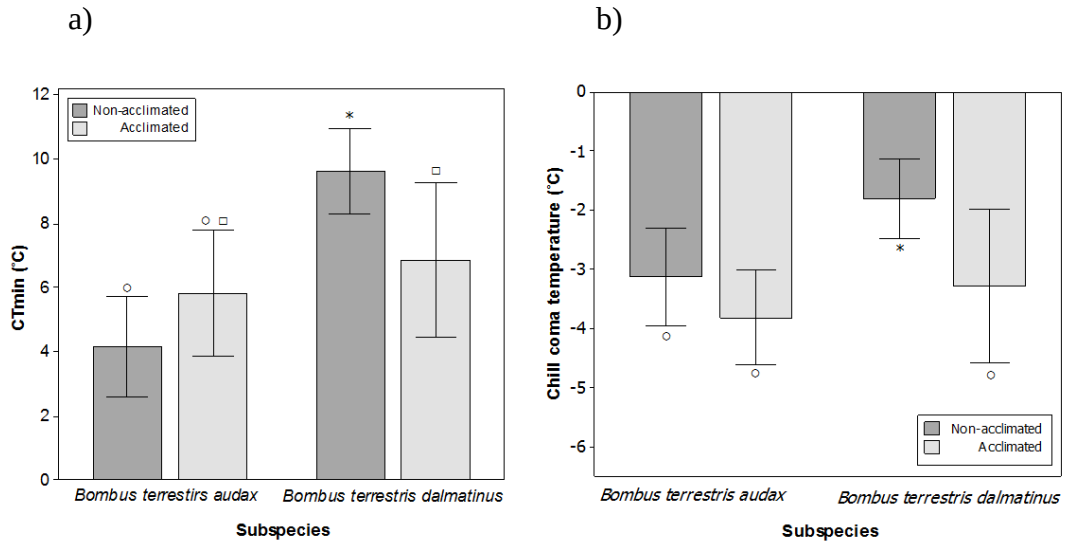


Figure 4.6 a) CTmin and b) chill coma temperatures of *Bombus terrestris audax* and *Bombus terrestris dalmatinus*, with a period of acclimation (7 days at 10°C) or without (rearing temperature 20°C). Means (with Bonferroni-corrected 95% confidence interval bars) are presented for n=15 bees for each treatment. Different symbols represent significant differences of p<0.05.

Chill coma recovery and activity recovery

The mean chill coma recovery (± SE) temperature for non-acclimated *B. t. audax* was 0.8 ± 0.2˚C and in acclimated bees, this value was 2.1 ± 0.1˚C (Figure 4.7a). In *B. t. dalmatinus*, the mean (± SE) chill coma recovery temperature for non-acclimated bees was 2.5 ± 0.2 and in acclimated bees this value was 2.0 ± 0.6˚C (Figure 4.7a). Acclimation significantly increased the recovery temperature of *B. t. audax*, but had no effect on *B. t. dalmatinus* (p=0.02 and p>0.1 respectively, Tukey's HSD test). When comparing non-acclimated subspecies, *B. t. audax* had a significantly lower chill coma recovery temperature than *B. t. dalmatinus* (p<0.01).

All bees tested showed evidence of chill coma recovery; however, some bees were not able to recover to a full state of coordinated activity. For example, bees were able to move appendages but were unable to walk. These bees suffered mortality within hours of experimentation. For *B. t. audax*, this occurred for 6 of the 15 non-acclimated bees and 1 of the 15 acclimated bees. For *B. t. dalmatinus*, all non-acclimated bees (n=15) showed activity recovery, but 11 of 15 acclimated bees did not recover to a full level of coordinated activity.

Using the surviving *B. t. audax* individuals (n=9 and 14 for non-acclimated and acclimated bees respectively), the mean (± SE) temperature at which non-acclimated bees regained activity was 8.8 ± 0.5˚C and the value for acclimated bees was 9.1 ± 0.3˚C (Figure 4.7b). Using the surviving *B. t. dalmatinus* individuals (n=15 and 14 non-acclimated and acclimated bees respectively) the mean (± SE) temperature at which non-acclimated bees regained

activity was 9.9 ± 0.2˚C and the value for acclimated bees was 9.0 ± 0.9˚C (Figure 4.7b). There were no significant differences between acclimation regimes or subspecies for this activity threshold (p>0.05 in all cases).

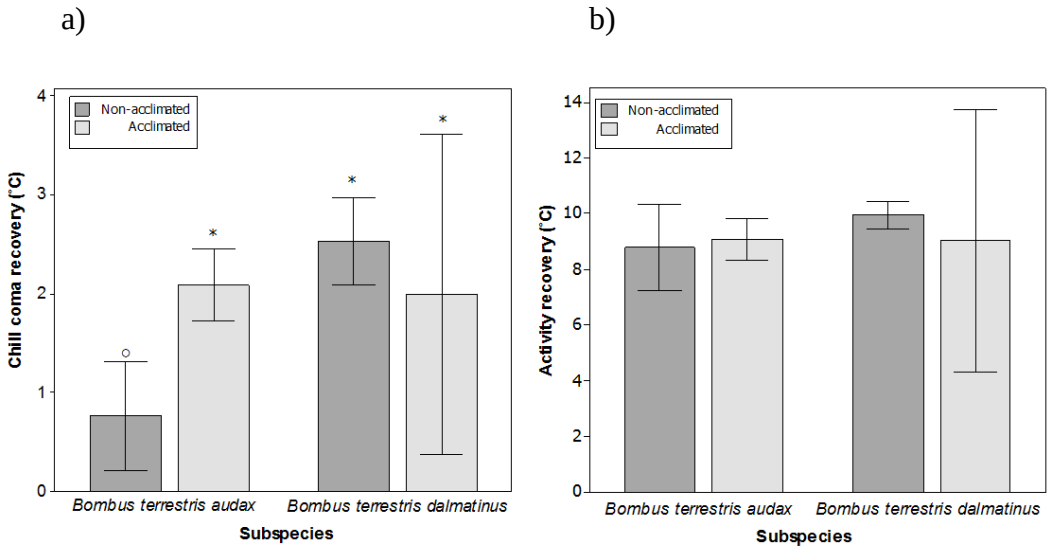


Figure 4.7 a) Mean (with Bonferroni-corrected 95% confidence interval bars) chill coma recovery and b) Mean (with Bonferroni-corrected 95% confidence interval bars) activity recovery temperatures of *Bombus terrestris audax* and *Bombus terrestris dalmatinus*, with a period of acclimation (7 days at 10°C) or without (rearing temperature of 20°C). Sample sizes for chill coma recovery experiments were consistent with other activity thresholds (n=15 bees per threshold). However, due to sub-lethal effects, not all bees regained a full state of activity recovery: only individuals who fully recovered were included in activity recovery analysis (for *B. t. audax*, n=9 and 14 for non-acclimated and acclimated bees; for *B. t. dalmatinus*, n=15 and 14 for non-acclimated and acclimated bees). Bars with different symbols represent significant differences of p<0.05.

CTmax and heat coma

The mean CTmax temperature for non-acclimated *B. t. audax* was 41.6 ± 0.8˚C, and 43.9 ± 0.4˚C for acclimated bees (Figure 4.8a). The mean heat coma temperatures for *B. t. dalmatinus* were 41.2 ± 1.3 ˚C and 43.8 ± 0.5˚C for non-acclimated and acclimated

respectively (Figure 4.8a). There were no significant differences between acclimation regimes or subspecies for this activity threshold (p>0.05 in all cases).

The mean (± SE) heat coma temperature for non-acclimated *B. t. audax* was 44.1 ± 0.7˚C, while the value for acclimated bees was 44.8 ± 0.3˚C (Figure 4.8b). The mean (± SE) heat coma temperatures for *B. t. dalmatinus* were 44.9 ± 0.9 and 47.5 ± 0.4˚C for non-acclimated and acclimated respectively (Figure 4.8b). Acclimation significantly increased the heat coma of *B. t. dalmatinus*, but had no effect on *B. t. audax* (p=0.03 and p>0.1 respectively, Tukey's HSD test). When comparing non-acclimated subspecies, there was no significant difference in heat coma temperature between non-acclimated *B. t. audax* and *B. t. dalmatinus* (p>0.1).

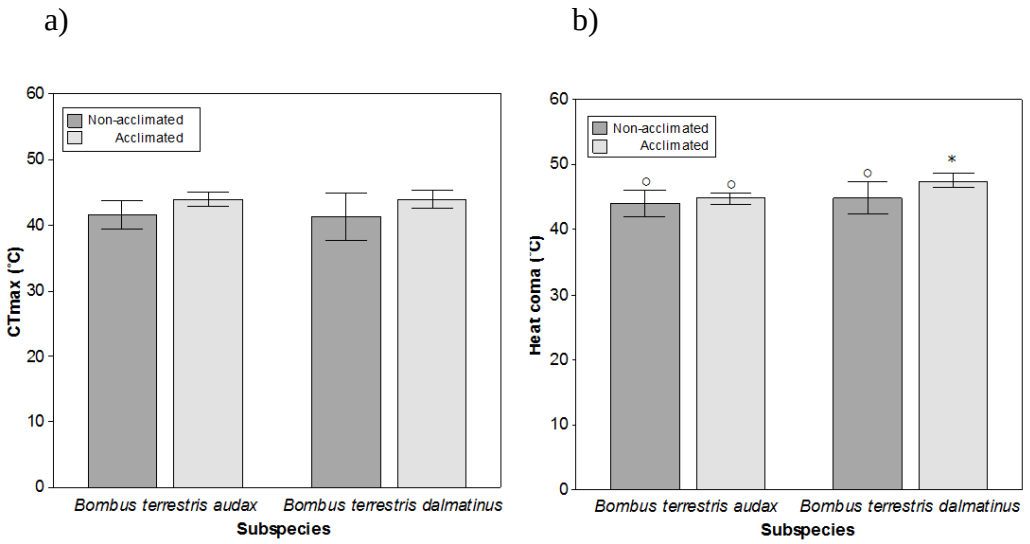


Figure 4.8 a) Mean (with Bonferroni-corrected 95% confidence interval bars) CTmax and b) Mean (with Bonferroni-corrected 95% confidence interval bars) heat coma temperatures of *Bombus terrestris audax* and *Bombus terrestris dalmatinus*, with a period of acclimation (7 days at 10°C) and without (rearing temperature of 20°C), n=15 for threshold. Bars with different symbols represent significant differences of p<0.05.

4.5. DISCUSSION

There is clear evidence that climate change is impacting the phenology of many insect species (Watt and McFarlane, 1991; Roy and Sparks, 2000; Hickling *et al*., 2005; Visser and Both, 2005; Parmesan, 2006; Bale and Hayward, 2010). The loss of synchrony between phenology and seasonal change brings an increased risk to insects, exemplified by winter-active bumblebees. Worker bumblebees are increasingly foraging at low temperatures; a phenomenon which not only risks individual mortality but can have a fundamental impact on colony growth and survival (Weidenmüller *et al*., 2002). This study has shown that the UK subspecies *B. t audax* is better adapted to forage at lower temperatures than the imported species *B. t dalmatinus*. However, both subspecies are poorly adapted to winter foraging given the close proximity of their lower activity thresholds to UK temperatures: for example, the mean daily temperature in March 2014, at the beginning of spring, was 2.1°C in Birmingham, UK (Chapter 5). This is below the CTmin of both *B. t. audax* and *B. t. dalmatinus*, recorded in this study.

The minimum threshold for coordinated movement (CTmin) is an ecologically relevant measure of an insect's ability to remain active at low temperatures (Hatherly *et al*., 2005). Historically, this index has been used in the assessment of candidate glasshouse biological control agents which are often sourced from Mediterranean or tropical regions specifically because they are unlikely to survive winter temperatures, and thus not establish in the UK (van Lenteren, 1997). Accordingly, many of these Mediterranean species have tended to have high CTmin values, and suffer mortality as a result of low temperatures. For example, the

mirid *Nesidiocoris tenuis* has a CTmin of 4.0 ± 0.1°C (Hughes *et al*., 2010). Interestingly the CTmin value of *B. t. dalmatinus* (also from the Mediterranean region) was even higher (9.6 ± 0.5˚C), suggesting very limited activity would be possible during UK winters. Somewhat surprisingly, the CTmin of the UK bumblebee subspecies *B. t. audax* was also high (4.2 ± 0.5˚C). Winter temperatures are frequently below 4°C between the months of October and May (Figure 4.9) in the UK midlands, and given that neither subspecies demonstrated evidence of endothermy it seems that commercial bees may not be able to forage under winter conditions. However, due to the short-term nature of these experiments, caution must be taken in attempting to determine long-term trends. These data represent a short term study and can be skewed by short-term variations in environmental temperature, such as unusually cold winters (*e.g*. winter 2010 in Europe; Cattiaux *et al*., 2010). Long-term monitoring over a period of ~30 years is needed to confirm if there is a survival risk to winter-active colonies.

Using 2012-2013 Birmingham temperatures as an example (Figure 4.9), July and August appear to be the only months in which temperatures below 9.6 ± 0.5°C (the CTmin of *B. t. dalmatinus*) were not experienced. These results suggest potentially big differences in the periods of activity open to non-native bees compared to the UK-native *B. t. audax*, which need to be confirmed in long-term studies. Whether this translates to differences in pollinator performance needs to be investigated further.

The worldwide trade in bumblebees is ongoing, with commercial bumblebee colonies imported at a rate of 60,000 per year to the UK (Goulson, 2010). Colonies are used to

pollinate crops in the open field, as far north as Scotland (Whitehorn *et al*., 2013). In order for bumblebees to pollinate efficiently, their body temperature must be above their CTmin, given that this is the threshold for coordinated movement. It follows, therefore, that the lower the ambient temperature, the lower the pollination efficiency. In areas of high latitude, and thus lower temperatures, foraging may cease completely as bumblebees are unable to move in a coordinated way. For example, bumblebees in Scotland may be exposed to air temperatures (Figure 4.9) below their CTmin more frequently than bumblebees in Birmingham (Figure 4.10). This has wide-ranging impacts upon the pollination industry, given that bumblebees may be producing sub-optimal pollination rates even during the summer pollination period.

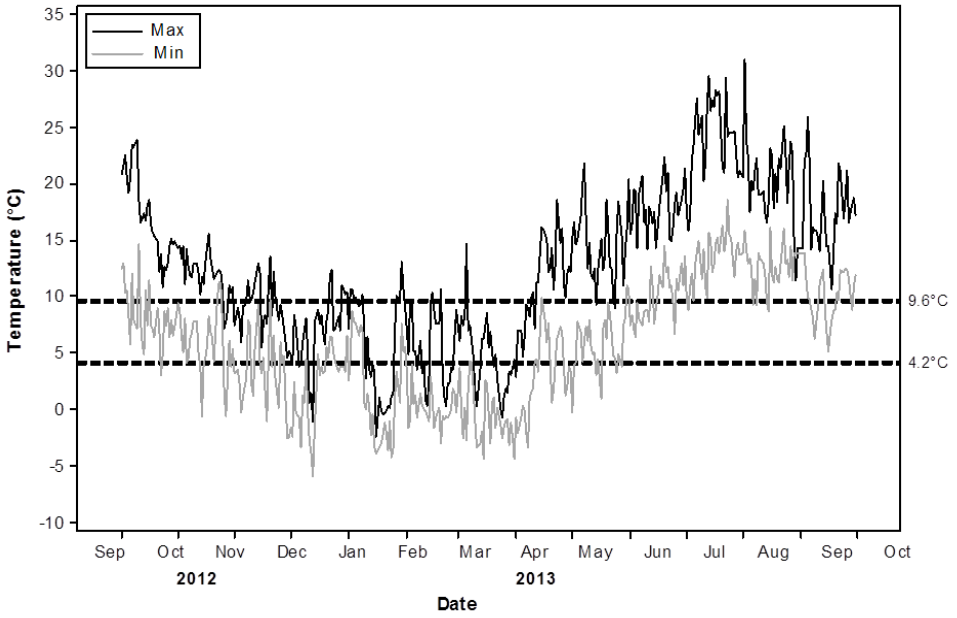


Figure 4.9 Minimum and maximum air temperatures recorded between September 2012 and October 2013 in Birmingham, UK. Overlaid on the y axis are two dashed lines; one at 9.4°C and one at 4.2°C, representing the mean CTmin temperatures in *B. t. dalmatinus* and *B. terrestris audax* respectively.

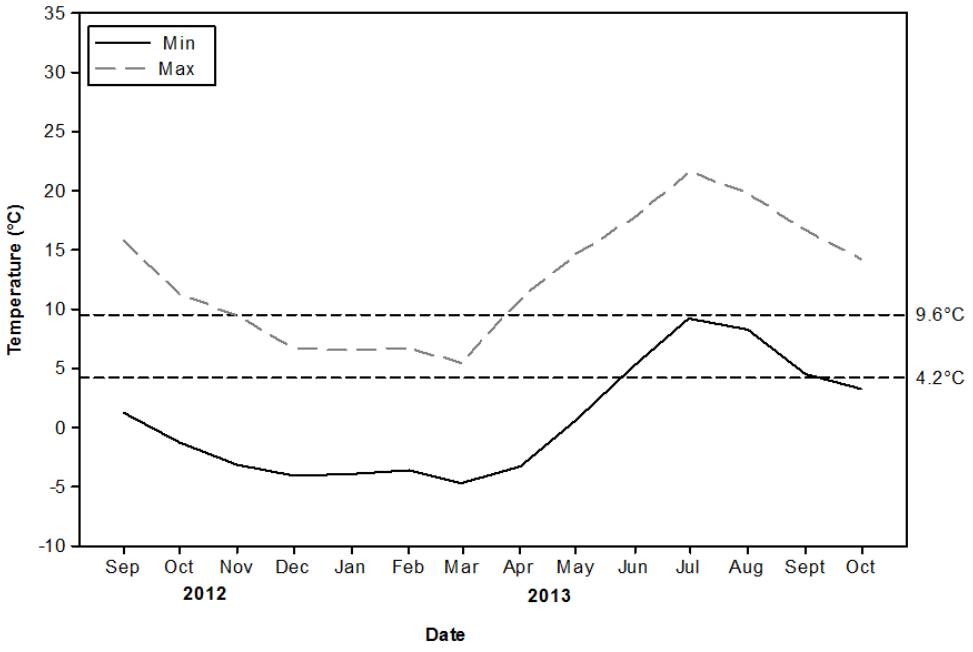


Figure 4.10 Minimum and maximum monthly air temperatures in Edinburgh, Scotland, obtained from the Edinburgh Royal Botanic Garden (2015). Overlaid on the y axis are two dashed lines; one at 9.4°C and one at 4.2°C, representing the mean CTmin temperatures in *B. t. dalmatinus* and *B. t. audax* respectively.

The endothermic capacities of bees have been widely reported. In most studies, as temperature decreases, metabolic rate increases as a result of increasing heat generation. Heat generation is needed to maintain a stable flight temperature when foraging and to maintain a high brood temperature within the colony (Heinrich, 1975). An inverse relationship between temperature and metabolic rate, as described above, was reported in the honeybee *Apis mellifera* (Harrison *et al*., 1996), the anthophorid bee *Centris pallida* (Roberts and Harrison, 1998) and the orchid bee *Euglossa imperialis* (Borrell and Medeiros, 2004). Bees were able to generate heat to counteract the decrease in ambient temperature. However, this is not the case in all bees; and it appears that the ability to forage and the availability of a floral incentive can also influence endothermic responses. For example, the alpine bee, *Andrena bicolor*, lacked any form of endothermy when tethered but did generate heat during free-flying foraging (Herrera, 1995). In agreement with this, Woods *et al*. (2005) reported endothermy only in free-flying bees, and not in bees requiring prompting or agitation to fly. Finally, Harrison and Fewell (2002) found low levels of heat generation in *Apis mellifera* individuals that were tethered, winter acclimated or those without a foraging-incentive. These studies suggest that endothermy depends on the perceived ability of a bee to obtain floral rewards, and so bees that are unlikely to forage due to tethering may not expend energy in generating heat. This may explain why both subspecies showed little evidence of endothermy (Figures 4.1 to 4.4), even at decreasing temperatures. This may be attributed to the tethered nature of the bumblebees (as they were attached to thermocouples) and the lack of free-flight and floral reward. It is unlikely to be an effect of using commercial bees for experimentation as wild bees tracked a similar trajectory and showed little sign of endothermy when tested (Figure 4.5). Even if endothermy were to occur under more natural conditions, however, different

CTmin thresholds for *B.t dalmatinus* vs *B. t. audax*, suggest the energetic costs of endothermy could be very different.

Chill injury is thought to occur as a result of phase changes in membranes and alterations to metabolism (Renault *et al*., 2004). Acclimation reduces the speed at which chill injuries are acquired (Renault *et al*., 2004) by minimising damage caused by oxidative stress (Rojas and Leopold, 1996). It provides the insect time to accumulate polyols and sugars, synthesise antifreeze proteins, and exclude ice nucleating agents (Bale, 1996). Worker honeybees are known to respond to periods of acclimation by lowering their chill coma temperatures (Free and Spencer-Booth, 1960) in line with other insect species such as the aphid *Myzus persicae* and the parasitic wasp *Lysiphlebus testaceipes* (Hughes *et al*., 2010; Alford *et al*., 2012). The results of our study show that *B. t. dalmatinus* was responsive to periods of both low (10°C) and high (30°C) temperature acclimation, with CTmin and chill coma temperatures significantly decreasing and heat coma temperatures significantly increasing as a result of acclimation. Interestingly, *B. t. audax* did not respond positively to acclimation. Indeed, acclimation had a negative effect on the chill coma recovery temperature of this subspecies (Figure 4.7a and b); perhaps because the acclimation temperature was low enough to cause chill injury.

During the warming stage of chill coma recovery experiments, after cold temperature exposure, all individuals of both subspecies appeared to exit chill coma and began to twitch. However, some individuals continued to twitch without regaining coordinated movement,

irrespective of temperature. These individuals died within hours of experimentation. This suggests lethal damage as a result of low temperatures, a factor which would render individuals unable to forage in the wild. Temperatures which induce freezing and result in cold mortality in *B. t. audax* and *B. t. dalmatinus* are discussed in Chapters 2 and 3. As these temperatures are comparatively high for subspecies active at low temperatures (mean SCP of *B. t. audax* was -7.1 ± 0.2˚C, mean chill coma temperature was 4.2 ± 0.5˚C), there is inevitably a level of crossover between temperatures causing chill coma and those resulting in freezing and mortality. Chill coma recovery and activity recovery experiments included temperatures which may have caused freezing or lethal damage to a small proportion of individuals, due to the close proximity of activity thresholds and fatal temperatures. This, however, was unavoidable, as a minimum temperature had to be selected which induced chill coma in 100% of individuals.

When considering the upper thresholds for activity, there were no significant differences between subspecies, with little variation between samples. After a period of acclimation, *B. t. dalmatinus* was the only subspecies to increase its heat coma temperature. Both *B. t. audax* and *B. t. dalmatinus* would not be predicted to encounter temperatures close to their CTmax in greenhouses and gardens throughout the native range of *Bombus terrestris*, however, flower temperatures can often approach these values on hot days in the UK. Flower temperatures at Winterbourne Botanic Gardens (52˚27’13”N 1˚55’29”W) in July 2013 recorded a mean (± SE) of 41.4 ± 1.5˚C, *n*=15 (personal observations). Given the pollination and foraging activity of both subspecies, this temperature may be encountered by bees in

summer months and may exceed the CTmax of both subspecies. This is important given how close CTmax values are to heat coma and upper thermal limits. Bumblebees are known to select flowers with higher temperatures when foraging to minimise heat loss and metabolic demand (Rands and Whitney, 2008). This suggests a capacity to detect flower temperature, therefore they may avoid flowers that are too warm. The fact that *B. t. dalmatinus* can increase its CTmax as a result of acclimation may prove a significant adaptive advantage to the subspecies in times of high temperature.

In conclusion, both *B. t. audax* and *B. t. dalmatinus* are likely to suffer extended periods of inactivity in UK winters, as a result of environmental temperatures being below their activity thresholds. Indeed, this study has shown that even temperatures in spring and autumn may be low enough to render bumblebees immobile. The native *B. t. audax* has been shown to remain active at lower temperatures, and may therefore be better adapted for activity in early spring and late autumn. However, the ability of *B. t. dalmatinus* to acclimate to low temperatures may result in direct competition between the subspecies, with unknown consequences.

CHAPTER 5

PHENOLOGY OF WILD *B. T. AUDAX* IN BIRMINGHAM AND THE CAPACITY FOR WINTER COLONY SURVIVAL

5.1. ABSTRACT

Recent evidence suggests bumblebees (*Bombus terrestris audax*) are becoming winter-active in the south of the UK. Numerous anecdotal reports suggest activity is possible in more northerly areas; however, little published evidence exists to support this. To determine the abundance of bumblebees (of all castes) in Birmingham, transect walks were carried out in an urban botanical garden. To determine the ability of winter-active colonies to survive at low temperatures, two experimental scenarios were replicated: 1. the aversion of diapause by queens and the initiation of a second, winter-active colony in autumn (Diapause Averted Colonies, DAC); 2. early termination of diapause by queens and the establishment of an early spring colony (Early Termination Colonies, ETC). The survival rate, foraging activity and pollen load of bumblebees were monitored for the duration of the colonies. Results showed an absence of wild, winter-active bumblebees. DAC colonies exhibited an initial 3-week period of activity, before a slow decline to colony death after ~8 weeks. Due to the low temperatures experienced in March 2013, ETC colonies showed no evidence of foraging activity and also collapsed after ~8 weeks. Both colonies were unable to maintain a constant colony temperature typical of successful colonies. The results of this study provide valuable information about how phenological changes are likely to impact on the survival and foraging abilities of a key pollinator species.

5.2. INTRODUCTION

In temperate zones, winter represents a significant challenge to many organisms, with low temperatures driving both individual and community-level changes (Williams *et al*., 2015). Insects have adapted to avoid or tolerate low temperatures (Bale, 1993), with strategies such as diapause becoming an important part of the life history and winter survival (Wadsworth *et al*., 2013). Climate warming is changing the nature of temperate winters (Kreyling, 2010), and this is, in turn, altering the phenology of many insect species (Schweiger *et al*., 2010). Aside from an increase in mean global temperature (IPCC, 2014), climate change is also altering the frequency of extreme cold events, the availability of water and the instances of frost and snow (Williams *et al*., 2015). These factors have a direct impact on insect overwintering, including their survival and the timing of life history events (Bale and Hayward, 2010).

Insects have responded to climate change in a variety of ways, with many recorded incidences of spatial and temporal shifts (Roy and Sparks 2000; Hickling *et al*., 2005; Parmesan, 2006). These shifts can also result in the loss of synchrony between animals and plants, which often respond in different ways to a change in environmental conditions (Williams *et al*., 2015). Of key importance to the provision of ecosystem services is the mismatching of pollinators and plants (Vanbergen *et al*., 2013; Petanidou *et al*., 2014; Polce *et al*., 2014), and there is increasing evidence that pollinators have undergone significant changes in abundance, range and phenology as a result of climate change (Memmott *et al*., 2007; Hegland *et al*., 2009; Schweiger *et al*., 2010; Fagan *et al*., 2014). One such example is the bumblebee *B. t. audax*, native to the UK (Stelzer *et al*., 2010). Typically a monovoltine species in northern Europe,

workers are usually present between the months of March and late September, after which colony death occurs (Heinrich, 1975; Goulson, 2010). At this point, the queen enters a state of dormancy, termed diapause, in which she remains until the following spring (Sladen, 1912). However, there have been recent sightings of worker bumblebees (Figure 5.1; Stelzer *et al*. 2010) and nest founding queens (Goulson, 2003) during winter in southern UK. This suggests two possible scenarios. First, queens may be averting diapause at the end of the summer months and establishing a new, winter generation - as has been recorded in other bumblebee species, such as *Bombus vosnesenskii* Radoszkowski, 1862 (Hymenoptera: Apidae) in North America (Skyrm *et al*., 2012). Second, queens could be entering a shorter diapause in late summer, which means they emerge in mid-winter and initiate colonies in late winter or early spring. Comparable phenological advances have been recorded in at least 10 North American bee species, with Bartomeus *et al*. (2011) suggesting there has been a mean advance in queen emergence of 10.4 ± 1.3 days since 1880 (based on a general linear mixed model). Thus, *B. t. audax* represents an interesting example of a British bumblebee altering its phenology which, in common with the North American species, is predicted to be a response to climate change (Stelzer *et al*., 2010).

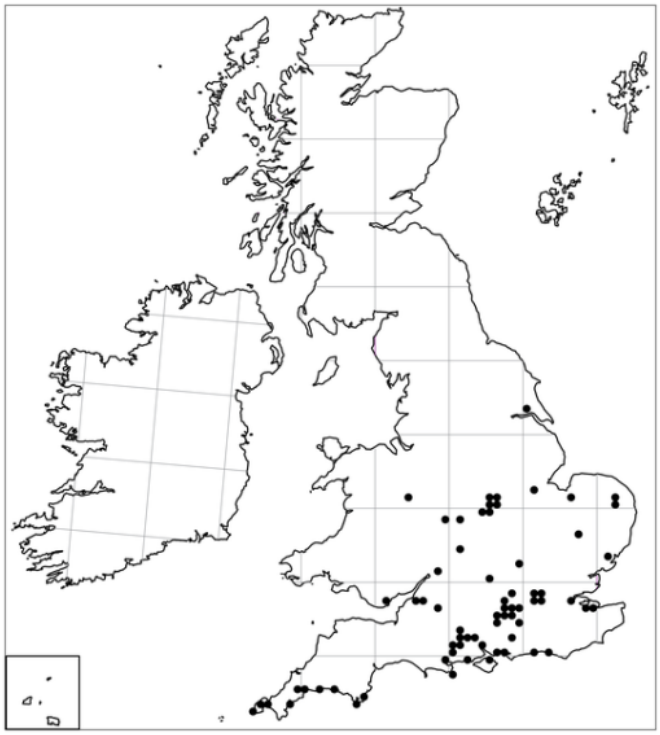


Figure 5.1 Figure and legend taken from Stelzer *et al*. (2010). Distribution of winter active *B. terrestris* in the UK from October 2008 to March 2009. Data (247 records of workers and 329 of queens) were kindly provided by the Bees, Wasps and Ants Recording Society and the map was produced by Stuart Roberts.

Very little is known about diapause in *B. t. audax*, though it is thought to be facultative (Beekman *et al*., 1999) and highly variable in duration in wild queens (Amin *et al*., 2011). While facultative diapause in temperate insects is predominantly induced by photoperiod (Denlinger, 2002), temperature plays an important role in determining both diapause incidence and duration. There are many examples of insects averting diapause under certain temperature conditions even under diapause-inducing short day conditions, for example, populations of the blowfly *Calliphora vicina* in the UK (Bale and Hayward, 2010). Even if diapause is induced, its duration can be dramatically shortened under warm conditions as energy stores are often utilised more rapidly (Hahn and Denlinger, 2007). Thus, as outlined

earlier, queens produced in late summer, under recent climate warming, could be either averting diapause or terminating diapause early. This has the potential to alter the characteristics of future colonies, with diapausing queens producing colonies with increased worker numbers and longevity, but fewer queens (Beekman and van Stratum, 2000). There is also the question of whether winter active bees are able to survive low temperatures (see Chapters 2, 3, and 4). It is important, therefore, to understand changing phenological patterns in UK bumblebees, as this is directly relevant to both their spatial and temporal abundance, and consequently the level of pollination service provision.

While the recently reported bivoltinism in *B. t. audax* in Kew gardens is a novel phenomenon for this subspecies, bivoltinism is common in another subspecies native to the Mediterranean, *B. t. dalmatinus*, where winter temperatures are high enough to sustain a second, winter generation (Gurel *et al*., 2008). Bivoltinism has also been reported in *B. terrestris* subspecies exported to non-native countries, such as New Zealand (Cumber, 1954; Goulson, 2010) and Tasmania (Buttermore, 1997). If a queen is to avert diapause and initiate a second (winter) colony, the workers she produces must collect sufficient pollen and nectar to sustain the colony until the following spring (Stelzer *et al*., 2010; Weidenmüller *et al*., 2002). Colonies must be thermoregulated to between 30 and 32˚C for successful brood development (Heinrich, 1975), which represents a huge foraging demand given winter temperatures in the UK. It follows, that successful winter colonies must have regular access to winter forage, and therefore may be restricted to urban parks and gardens (Goulson *et al*., 2008, Stelzer *et al*., 2010).

To date, the only study on winter-active bumblebees has been undertaken in the south of the UK (Stelzer *et al*., 2010). As *B. t. audax* is a commercially valuable pollinator (Velthuis and van Doorn, 2006) which is used UK-wide (Biobest, 2015), it is important to document the occurrence of winter activity at a more northerly location, which will help towards determining the scale of winter activity in this subspecies. It is also important to investigate the capacity of colonies to survive winter conditions under the two scenarios set out above. For example, colonies established in late autumn (diapause averted), and colonies established in late winter/early spring (diapause terminated early). This study sought to investigate these issues by conducting field experiments in Birmingham, UK. Wild bumblebee abundance was monitored, in addition to the placement of bumblebee colonies in the field. Impacts on the abundance and distribution of *B. t. audax*, as well as the wider implications on pollination service provision are discussed.

5.3. METHODS

Transect walks

To document the winter activity of *B. terrestris* in late autumn, winter and early spring in Birmingham, transect walks were undertaken at Winterbourne Botanical gardens (52˚27’13”N 1˚55’29”W), Birmingham UK between February 2012 and March 2014. In total, 193 fixed transect walks were performed between 11:00h and 14:00h, using similar methods to Stelzer *et al*. (2010). Briefly, each walk followed a fixed route of approximately 750m lasting ~25min. The route passed approximately 9 flowering beds, containing a range of flowering

plants, depending on the season (see Appendix A). The number of *B. terrestris* were counted in each flower bed, and their caste was noted. If there were no bumblebees observed immediately, the flower bed was observed for 1 minute before the walk was resumed. Transect walks were not conducted in rainy conditions as bumblebees are known to avoid flying in wet conditions (Goulson, 2005). Mean monthly temperature and rainfall data were supplied by a weather station in Edgbaston, Birmingham, UK, for the entirety of the data collection period.

Experimental colonies

In the winter of 2012/2013, commercially-reared *B. t. audax* colonies were housed in Winterbourne Botanical Gardens, in Birmingham, UK. Experiments were designed to replicate two scenarios: 1. queens avert diapause and establish a second colony in autumn. In this scenario, colony survival and foraging performance were recorded in mature colonies, placed in the field on 2nd October 2012 (n=3, containing 40+ workers). 2. queens terminate diapause early and establish a new colony in early spring. In this case, immature colonies were placed in the field on 19th March 2013 (n=4, containing approximately 10 workers).

Colonies of *B. t. audax* were obtained from Biobest (Belgium) and were housed in plastic boxes (for waterproofing). Boxes were insulated, with polystyrene and cotton to simulate the thermal buffering provided by subterranean microhabitats. Entry and exit of bees was via a transparent tube extending from the brood chamber to the outside of the colony. Colonies were supplied with a small amount of pollen and nectar for the first few days, after which they

would be required to forage in order to sustain the colony. To measure the temperature within the colony, a TinyTag® data logger (with a sampling interval of 1h) was inserted into each colony. Temperature data were downloaded to a computer, using TinyTag Explorer® software, at frequent intervals. This data was used to provide mean daily temperatures inside each colony, which could be compared to weather station temperature data.

Data collection

Between 11:00h and 14:00h (times chosen to reflect bumblebee peak activity period), biweekly, the number of occupied brood cells, number of live workers and brood area of each colony were recorded. Additionally, 'colony traffic' was monitored for 1h, recording the number of workers entering (traffic 'in') and exiting (traffic 'out') the colony (see Kwon and Saeed, 2003; Molet *et al.*, 2009). Additionally, the presence/absence of a pollen load on the corbiculae of returning foragers was noted (see Dramstad, 1996), and the number of bees returning with a pollen load was recorded. This was used to estimate the number of successful foraging trips accomplished by workers and their capacity for winter foraging activity. Flowering plants were abundant throughout the duration of the experiment, providing sufficient forage (see Appendix A), and bumblebees were observed using a wide range of flowers. Colonies were removed from the field when all workers and the queen suffered mortality.

Statistical Analysis

A number of preliminary statistical tests were undertaken on the response variables to determine whether parametric or non-parametric testing could be employed. Normality was tested using Kolmogorov-Smirnov tests; equality of variance (homoscedasticity) was investigated using Levene's tests and time series were plotted to confirm the independence of samples. All response variables in this chapter were not normally distributed, but were homoscedastic and independent, therefore Mann-Whitney U tests were undertaken in SPSS®.

5.4. **RESULTS**

Winter activity in wild B. t. audax populations

Queen bumblebees were observed in 19 of the 27 months, with the highest monthly mean of queens (3.3 ± 0.7) recorded in October 2012. No queens were recorded in December or January during the entire observation period (Figure 5.2).

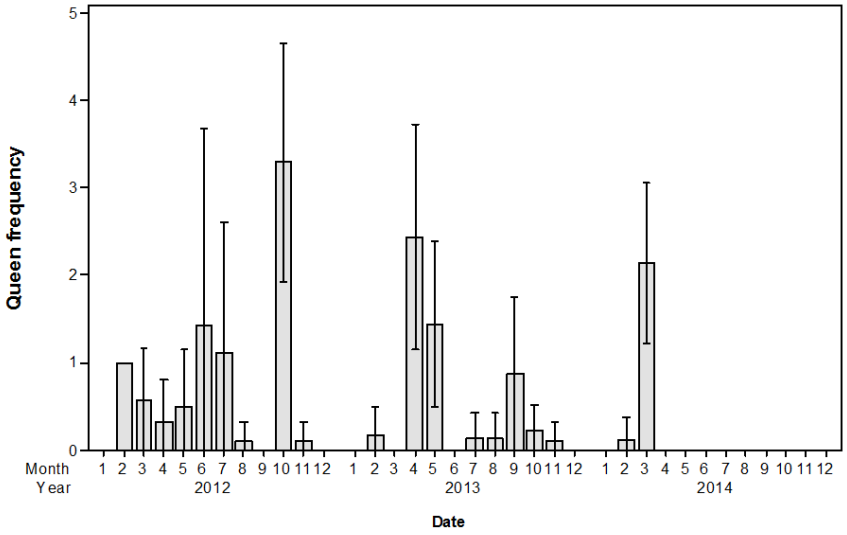


Figure 5.2 Number of *Bombus terrestris audax* queens (±2SE) recorded on transect walks at Winterbourne Botanical Gardens, Birmingham, UK, from January 2012 to March 2014.

Worker bumblebees were observed in 15 of 27 months, throughout the period of observation (Figure 5.3). The highest number of workers was recorded in June 2012 (92.1 ± 33.2). Sightings occurred exclusively within the March-October activity window, with winter activity being undetected in this study. The mean number of males recorded in the study was consistently low, with a peak of 3.7 ± 2.0 males sighted in September 2013 (data not shown). Males were exclusively recorded between the months of August and October.

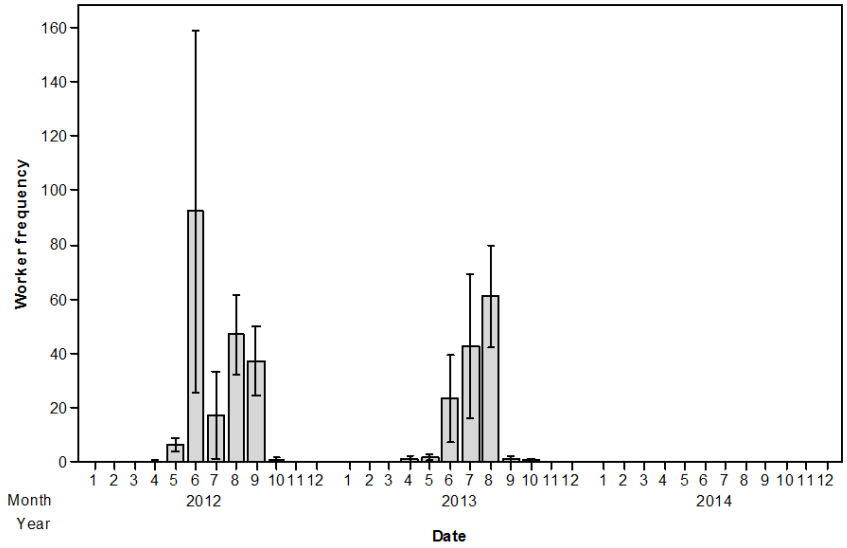


Figure 5.3. Number of *Bombus terrestris audax* workers (±2SE) recorded on transect walks at Winterbourne Botanical Gardens, Birmingham, UK, from January 2012 to March 2014.

Of key importance to this study, are the environmental conditions at the beginning of winter and the end of summer. At the beginning of winter 2012, temperatures in October ranged from -0.6 to 13.5°C compared to 2.9 to 20.2°C in October 2013 (Figure 5.4). At the end of winter 2013, temperatures in March ranged from -4.31 to 14.7°C, compared to -0.6 to 20.5°C in 2012 and -2.5 to 18.0°C in 2014. Temperatures in March 2013 appeared to be unusually low, and would have affected the activity of bumblebees in the field.

The 2013/2014 winter was in stark contrast to the previous year: mild temperatures (Figure 5.4) with high rainfall (Figure 5.5). At the beginning of winter, in October 2013, the amount of rainfall recorded was 136.8mm, compared to 67mm in October 2012. At the end of winter 2013, total rainfall in March was 66.4mm, compared to 20.6mm in 2012 and 2.6mm in 2014.

The high rainfall may have been responsible for the lack of queens in June or July 2013, in contrast to the previous year. However, this may have also been the result of colonies being established later in the year, due to the low March 2013 temperatures. The mean rainfall for 2012 was 85.9 ±14.3mm, for 2013 it was 63.52 ± 8.41mm, and for the first 3 months of 2014 (after which data collection ceased) it was 98.7 ± 32.2mm.

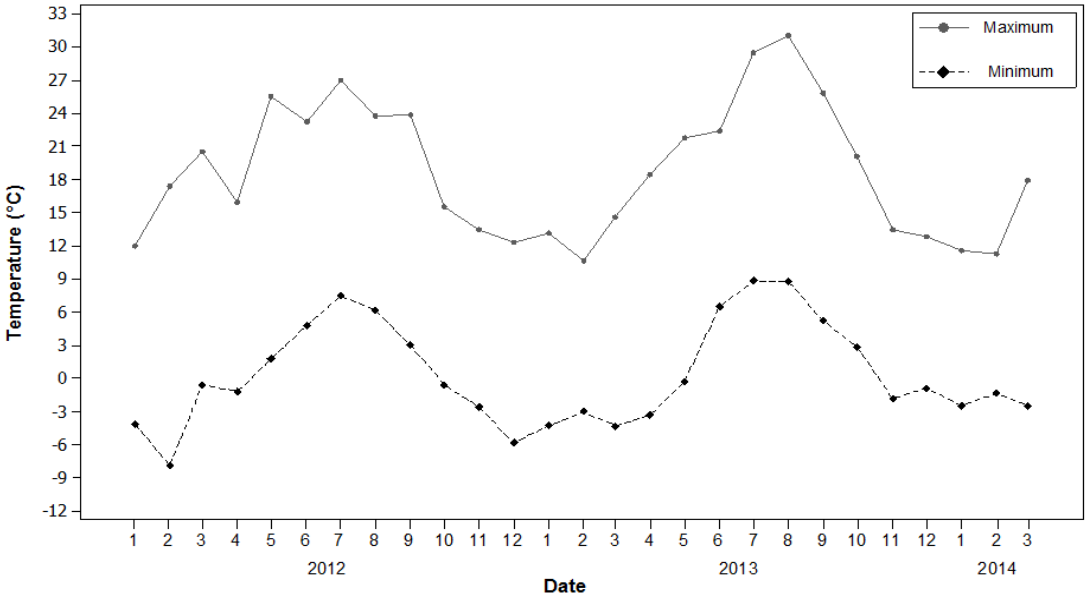


Figure 5.4.Minimum and maximum temperatures recorded in Edgbaston, Birmingham, UK from January 2012 to March 2014 inclusive.

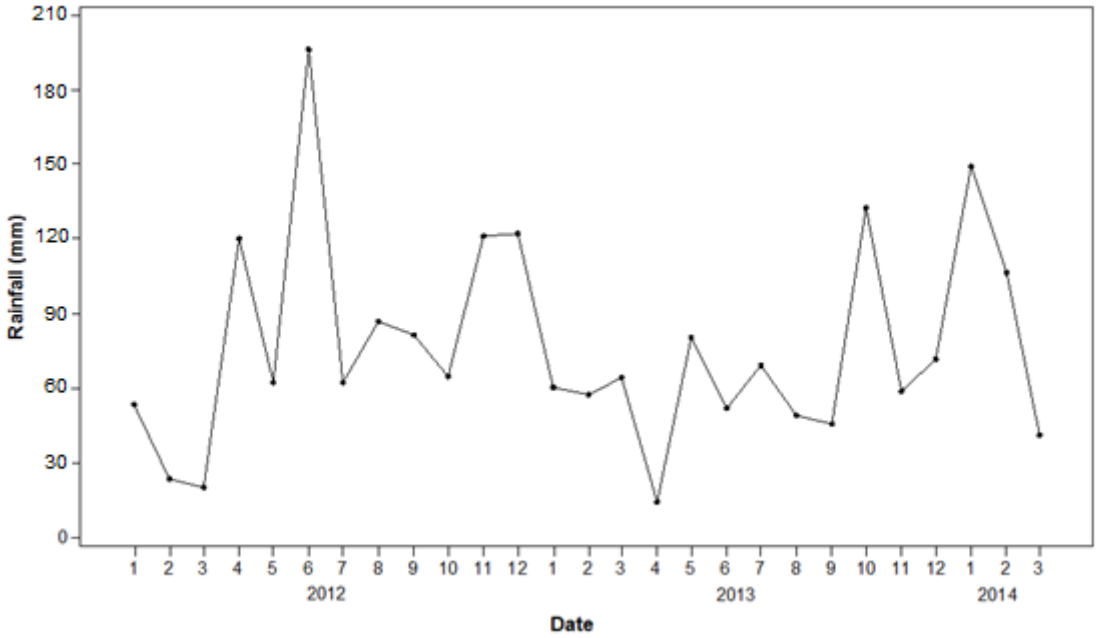


Figure 5.5.Total monthly rainfall in Edgbaston, Birmingham, UK, from January 2012 to March 2014 inclusive.

Scenario 1: Simulating colonies produced by queens averting diapause

Diapause Averted Colonies 1, 2 and 3 (DAC1, DAC2 and DAC3) started with 13, 19 and 24 individuals respectively (Figure 5.6). All colonies initially increased worker numbers to a maximum of 38, 33 and 43 workers after 2, 3 and 2 weeks in the field respectively. DAC1 began to decline after 30th October, with only 1 surviving individual remaining on 6th October. DAC2 fluctuated between 18 and 37 workers until 13th November, after which a rapid decline occurred, with 5 surviving individuals on 16th November. DAC3 survived the longest, with worker numbers fluctuating between 25 and 43 individuals until 27th November, after which worker numbers declined (Figure 5.6). On 30th November, there were 24 surviving individuals; however, there were no survivors by 4th December. Queen death occurred on the 28th November, 3rd December and 6th December in DAC 1, 2 and 3 respectively. The queens therefore outlived all workers in their respective colonies.

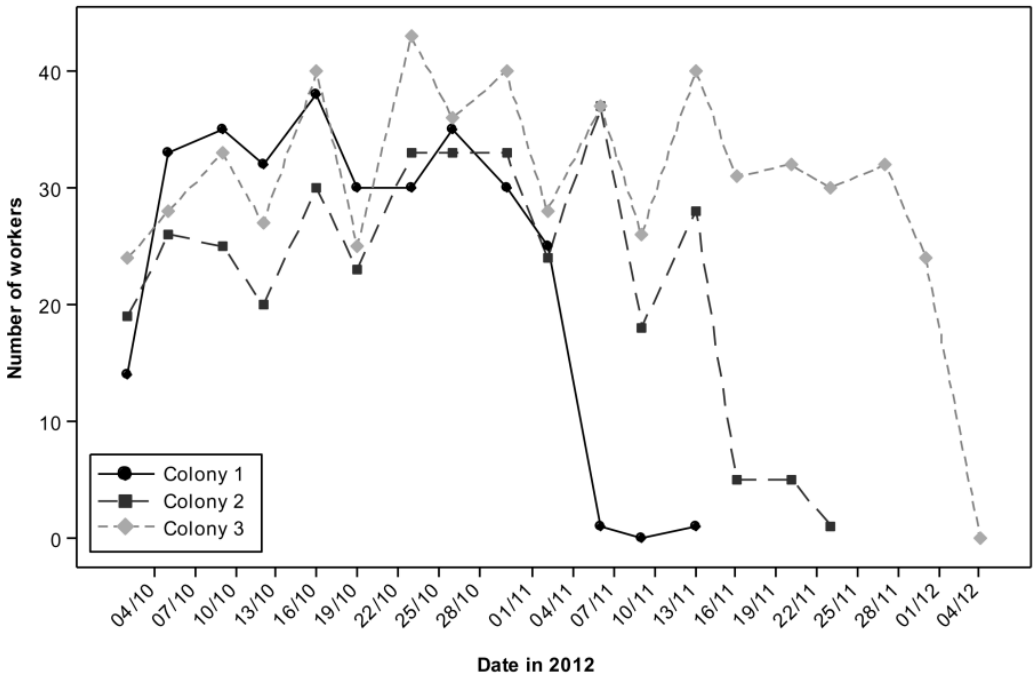


Figure 5.6. Number of live *Bombus terrestris audax* workers present in experimental Diapause Averting Colonies at Winterbourne Botanical Gardens, Birmingham, UK.

Only DAC1 and 2 recorded colony traffic (Table 5.1), as bumblebees in DAC3 did not enter or exit the colony during the sampling period. In DAC1, traffic peaked on 19th October 2012, with 90 individuals recorded entering the colony and 75 leaving the colony. Colony traffic ceased on 23rd October 2012. In DAC2, the highest number of bees entering the colony was on 19th October (41 workers), and the highest number exiting was on 16th October (43 workers). When comparing traffic between colonies 1 and 2, colony DAC1 had a greater amount of traffic, but the traffic from DAC2 spanned a longer period of time. When comparing colony traffic between colonies 1 and 2 using a Mann Whitney U test, no significant difference was found ($p>0.05$, $n=26$, U=89.5, W=180.5, se=18.9).

Table 5.1 Total number of *Bombus terrestris audax* workers entering ('Traffic in') and exiting ('Traffic out') Diapause Averting Colony 1 (DAC1) and Diapause Averting Colony (DAC2) during 1h observation periods at Winterbourne Botanical Gardens, Birmingham, UK.

	DAC1		**DAC2**	
Date	**Traffic in**	**Traffic out**	**Traffic in**	**Traffic out**
02/10/2012	2	2	2	23
05/10/2012	21	20	22	25
09/10/2012	32	25	28	15
12/10/2012	37	33	27	26
16/10/2012	48	46	25	43
19/10/2012	90	75	41	35
23/10/2012	20	17	35	1
26/10/2012	0	0	18	19
30/10/2012	0	0	4	0

Pollen loads of returning workers

Some of the workers returning to DAC1 and 2 (Table 5.1) were carrying a pollen load, suggesting a successful foraging trip. At the peak of their foraging activity (19th October), 17 workers from DAC1 returned with a pollen load (Table 5.2). In DAC 2, a peak of 14 workers also occurred on 19th October (Table 5.2). The numbers of workers returning with a pollen load was not significantly different between DAC1 and DAC2 ($p>0.05$, $n=20$, $U=55$, $W=100$, $se=12.5$).

Table 5.2 Total number of *Bombus terrestris audax* workers returning with a pollen load to Diapause Averting Colony 1 (DAC1) and Diapause Averting Colony 2 (DAC2) during 1h observation periods at Winterbourne Botanical Gardens, Birmingham, UK.

Date	DAC1	DAC2
02/10/2012	0	0
05/10/2012	13	4
09/10/2012	15	7
12/10/2012	14	9
16/10/2012	2	0
19/10/2012	17	14
23/10/2012	0	0
26/10/2012	0	0
30/10/2012	0	2

Temperature data for DAC experiment

Environmental temperature: When the colonies were placed in the field (2nd October 2012), the maximum temperature outside the colonies was 14.9°C and the minimum was 9.3°C (Figure 5.7). Temperatures gradually decreased throughout the duration of the experiment. When colonies were removed from the field on 6th December 2012, with no surviving workers, the maximum temperature was 2.0°C and the minimum was -4.1°C (Figure 5.7). The first sub-zero temperature occurred on 15th October 2012 (-0.62°C).

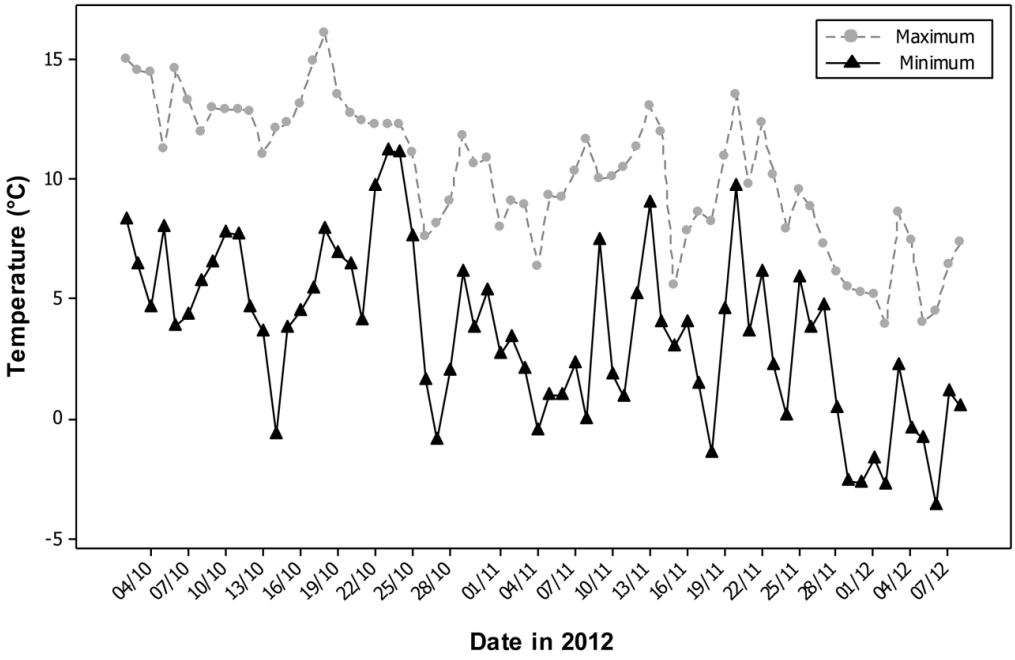


Figure 5.7. Minimum and maximum air temperatures at Winterbourne Botanical Gardens, Birmingham, UK.

Internal colony temperatures: When the colonies were first placed in the field (2nd October 2012), the minimum and maximum respective temperatures recorded inside colony 1 were 17.8 and 23.6°C, inside colony 2 were 18.8 and 24.1°C and inside colony 3 were 16.9 and 23.5°C (for the full data, see Appendix B). The minimum and maximum temperatures throughout the life of the colony are listed in Table 5.3.

Table 5.3 Minimum and maximum daily temperatures (and dates they occurred) of three *Bombus terrestris audax* colonies, placed outside in Winterbourne Botanical Gardens, Birmingham, UK between 02/10/2012 and 06/12/2012.

Colony	Minimum	Date of minimum	Maximum	Date of maximum
1	0.6	27/10/2012	23.7	03/10/2012
2	-0.8	18/11/2012	27.1	06/10/2012
3	-3.8	29/11/2012	22.1	03/10/2012

Scenario 2: Simulating colonies produced by queens terminating diapause early

After placement in the field (19th March 2013), all early termination colonies (ETC) continued to produce workers. ETC1 and 3 both increased from 9 workers to a maximum of 14 workers after 22 and 15 days respectively (Figure 5.8). ETC2 and ETC4 started with 5 and 14 workers respectively, and both increased by just 1 individual, after 11 and 15 days in the field respectively (Figure 5.8). Numbers began to decrease in the following days, with a decrease in worker numbers in ETC 1, 2, 3 and 4 on 13th, 3rd 6th and 10th April respectively. Colony collapse and complete mortality occurred first in ETC2 on 12th April, ETC3 on 19th April, ETC1 on 3rd May and ETC4 on 10th May 2013. Queen death occurred for ETC1 and 2 on 26th March (1 week after placement in the field), for ETC3 on 22nd March (3 days after placement) and for ETC4 on 2nd April 2013 (16 days after placement). There was no Colony Traffic recorded for any of the colonies in this experiment. There are therefore no pollen load data available.

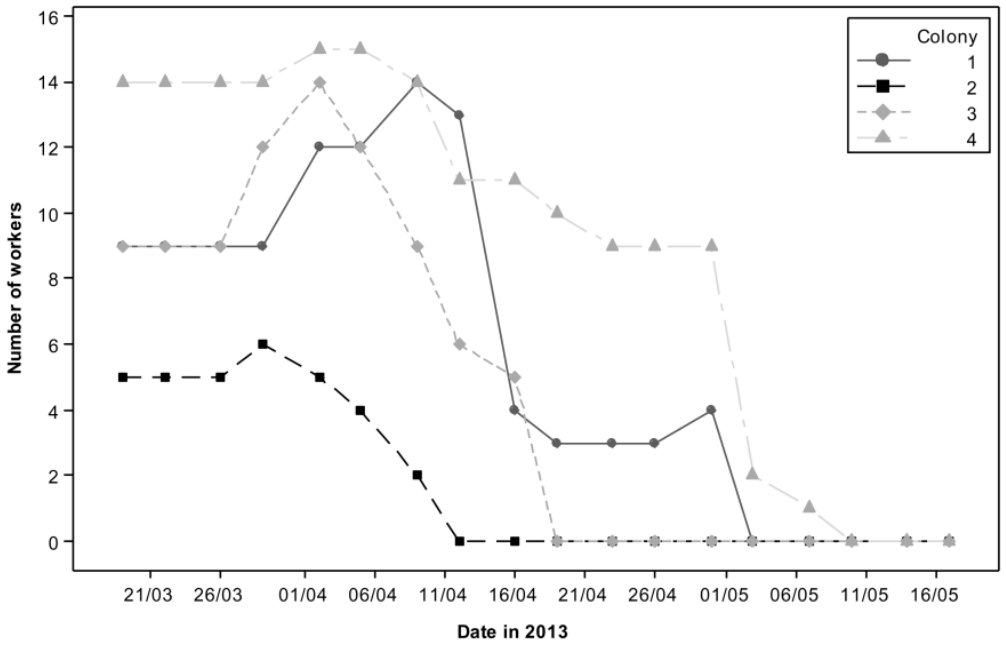


Figure 5.8. Number of live *Bombus terrestris audax* workers present in experimental Early Termination Colonies at Winterbourne Botanical Gardens, Birmingham, UK.

Temperature data – for ETC experiment

Environmental temperature: When the colonies were housed in the field on 19th March 2013, the maximum daily temperature recorded was 5.0°C and the minimum was 0.7°C (Figure 5.9). Temperatures gradually increased throughout the duration of the experiment. When colonies were removed from the field on 17th May 2013, with no surviving workers, the maximum temperature was 12.4°C and the minimum was 7.2°C (Figure 5.9). The last sub-zero temperature occurred on 1st May 2013 (-0.31°C).

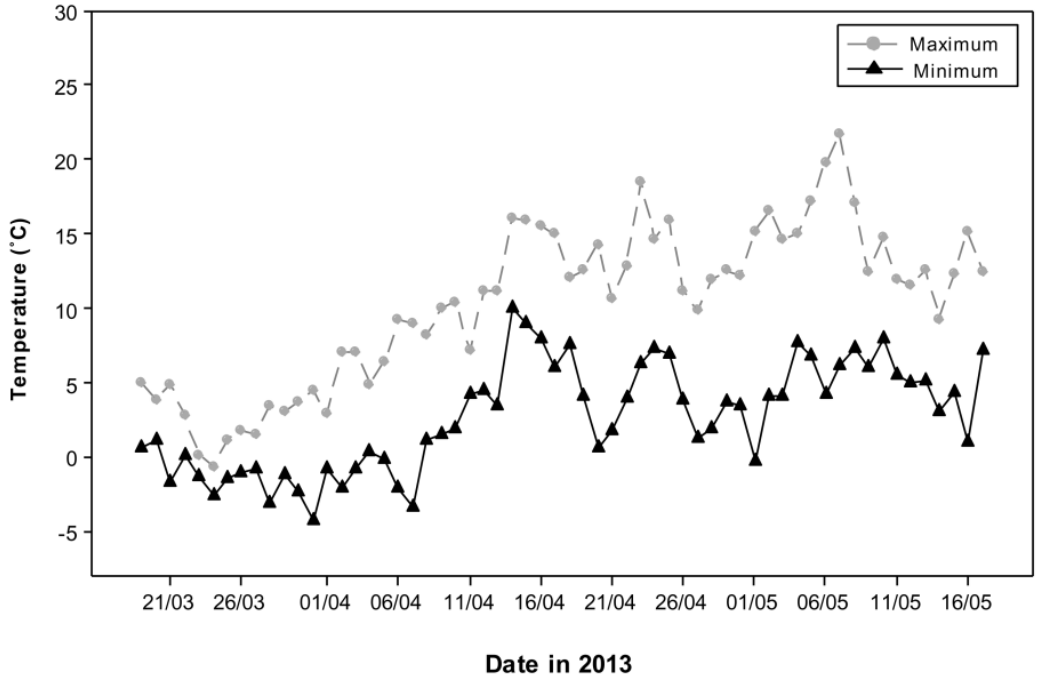


Figure 5.9. Minimum and maximum air temperatures at Winterbourne Botanical Gardens. Data provided by Duick Young, GEES, University of Birmingham, UK.

Internal colony temperatures: When the colonies were first placed in the field (19th March 2013), the minimum and maximum respective temperatures recorded inside colony 1 were 2.0 and 8.1°C, inside colony 2 were 2.2 and 7.3°C, inside colony 3 were 1.8 and 6.7°C and for colony 4 were 3.1 and 7.7°C (for the full data, see Appendix B). The minimum and maximum temperatures throughout the life of the colony are listed in Table 5.4.

Table 5.4 The minimum and maximum daily temperatures (and dates they occurred) of three *Bombus terrestris audax* colonies, placed outside in Winterbourne Botanical Gardens, Birmingham, UK, between 19/03/2013 and 17/05/2013.

Colony	Min. temp. (°C)	Date of minimum	Max. temp. (°C)	Date of maximum
1	-4.6	31/03/2013	28.0	20/04/2013
2	-3.6	31/03/2013	32.7	07/05/2013
3	-3.3	31/03/2013	27.9	07/05/2013
4	-4.3	31/03/2013	25.8	07/05/2013

5.5. DISCUSSION

This study has investigated the occurrence of winter activity in wild *B. t. audax* in Birmingham to determine if a bivoltine life cycle might be occurring further north than documented by Stelzer *et al*. (2010). In addition, this study placed commercial colonies in a field setting to investigate the survival capacity of winter active colonies established by either queens averting diapause, or, queens terminating diapause early.

B. t. audax phenology

When comparing results of transect walks in Birmingham with those undertaken by Stelzer *et al*. (2010) in Kew Gardens, London (Figure 5.10), differences between datasets are clearly apparent. Firstly, queens are sighted consistently throughout the year in Kew Gardens,

including the months of December and January (Figure 5.10). In Birmingham, queen sightings were completely absent in these months.

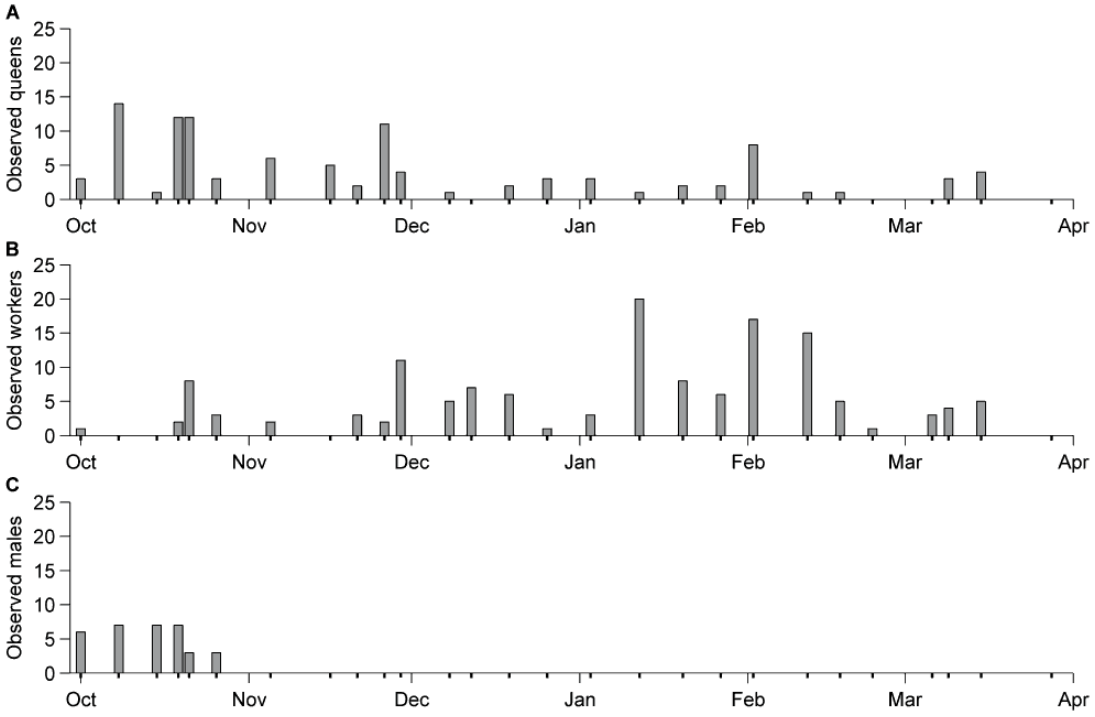


Figure 5.10, Figure and legend taken from Stelzer *et al*. (2010). *Bombus terrestris* activity during the winter at Kew Gardens (London, UK). Grey bars represent the number of *B. terrestris* queens (A), workers (B) and males (C) observed during the 27 transect walks conducted during the winter of 2007/2008. Individual transect dates are indicated by bold tick marks on the horizontal axis: six in October, five in November and four in each of the other months.

Workers in the current study (Birmingham) were exclusively found in the March-October activity window, typical of the univoltine bumblebee life cycle, whereas there were continuous sightings of workers throughout the year in Kew gardens. The presence of queens in June and July 2012 was unusually early (Figure 5.2), especially as there is a later peak in October, which is more characteristic of the species (Sladen, 1912). This cohort appears to be in diapause until April the following year, in contrast to the 9 month diapause duration suggested by Alford (1969). This may be due to the unusually low temperatures of early 2013

delaying the emergence from diapause, with March temperatures ranging between from -4.31 and 14.7°C.

In the Birmingham study, there were also large differences in queen abundance between 2012 and 2013, which may be attributable to differences in environmental conditions (Figure 5.2 and 5.4). The start of both 2012 and 2013 had lower than average temperatures (Met Office, 2014) and this cold spell extended until the end of March in 2013. Such a particularly cold and protracted winter in early 2013 may explain why the first queens were not seen until April - noticeably later than in 2012 (February) and 2014 (March). Although this data provides an interesting example the response of an insect's emergence in relation to temperature, a long-term study (over a 30-year period) would be needed to confirm a significant trend.

Diapause duration and the timing of emergence are known to be governed by temperature in many insects (Bale and Hayward, 2010), and *B. t. audax* emergence has been previously linked to soil temperature (Alford, 1969). Thus, diapause is likely extended during cold winters and queen emergence delayed until temperatures become more favourable. This can be a risky strategy, as longer diapause is associated with a greater metabolic cost and a decrease in queen survivorship (Beekman *et al*., 1998b). Also, the subsequent colony will have a shorter growing season, and may not produce as many queens for the following winter.

The amount of rainfall during this study was highly variable, and is likely to have affected the results. For example, in March 2013, at the start of the spring/summer experiment the level of rainfall was higher than March 2012 and March 2014 (Figure 5.5). Bumblebees are known to

avoid flying in wet conditions, as rain droplets impede pollen harvesting for corbiculae transportation (Peat and Goulson, 2005). This may have been responsible for the lack of colony traffic during this period. In times when rainfall was low, for example in October 2012 (with 67mm recorded during this month in Birmingham), more queens were observed in this month than any other in 2012, and more than October the following year. If bumblebees avoid foraging in wet conditions for extended periods of time, this can have a negative impact upon the amount of forage returned to the colony, perhaps impacting upon colony survival (Weidenmüller *et al*., 2002).

The early emergence of queens in 2012 (February) is followed by the presence of summer active queens in this year (June and July). It could be that early established colonies reached the point of queen production by mid-summer; however, this did not appear to result in any late/winter active colonies, as there is no evidence of worker activity beyond October, and the peak in queen production coincides with this decline in worker sightings. This all points to a more 'typical' colony seasonal cycle of colony death in mid-autumn coinciding with queens seeking overwintering sites. It is possible that queens may have averted diapause and attempted to initiate a winter-active colony, however, poor environmental conditions would then have prevented workers from leaving the colony and being recorded by our study. For example, worker bumblebees may have been physiologically unable to maintain flight at low temperatures (see Chapter 4) or because they were avoiding wet conditions (Peat and Goulson, 2005). The late emergence of queens in 2013 has been discussed earlier, and this explains the lack of any summer active queens during this year.

***Field experiments: Simulating diapause averting colonies* (*DACs*)**

Mature colonies placed in the field in October were able to continue producing workers for up to 3 weeks. The increase in numbers here can perhaps be attributed to the emergence of the brood first initiated in favourable temperatures within the lab, before the translocation of the colonies to the field site. This is supported by the fact that the typical development time for workers in the field is 4-5 weeks (Goulson, 2010). The fact that workers were still being produced at low temperatures contradicts studies reporting a necessary brood development temperature of 30-32˚C (Heinrich, 1975). It must be noted, however, that development would be predicted to be much slower at low temperatures, as is the case in most insects (Bale, 2002). After 3 weeks in the field, worker numbers decreased, with all colonies collapsing by 6th December. When comparing the temperatures recorded inside and outside colonies (Appendix B), there was little evidence of colony thermoregulation and heat generation. The inability to maintain a constant colony temperature has been associated with the collapse of the colony (Weidenmüller *et al*., 2002): this was the case for the colonies examined in this study.

Based on the low temperature tolerance of *B. t. audax* (Chapter 2) and the climactic conditions recorded during the field experiments (Figure 5.4), it seems unlikely that workers suffered extensive cold mortality. However, after the small amount of pollen and nectar initially supplied with the colonies (as in Ings, 2006) had run out, workers would be required to forage to prevent colony starvation (Whitehorn *et al*., 2009). Temperatures in October 2012

were frequently lower than the activity thresholds of this subspecies (Chapter 4), suggesting possible immobilisation of workers and a lack of forage returned to the colony. In honeybees, a lack of forage can result in a reduced immuncompetence and a reduction in adult survival and brood development rates (Naug, 2009), which can lead to a rapid collapse of the colony. This appears to be the case in our study. During colony observations it also became apparent that many underdeveloped brood had been evacuated from their cells and had perished on the outskirts of the colony. As bumblebee queens produce eggs at a constant rate during the linear phase of the colony (Beekman *et al*., 1998a), the queen may not cease egg laying as a result of low temperatures. However, as forage became limited in our study, live workers appear to have sacrificed the brood they could not feed. This appears to be the first report of such behaviour.

In honeybees, starvation also increases the number of workers recruited to foraging activity (Schultz *et al*., 1998). This is perhaps why colony traffic gradually increased in DAC1, after colony resources had depleted (Table 5.1). Interestingly, however, the number of successful foraging trips (Table 5.2) decreased in DAC1 over time. This could be because the environmental temperature gradually decreased throughout the duration of colony's placement in the field, and lower temperatures result in less efficient foraging (Kwon and Saeed, 2003).

Workers from DAC3 were not observed outside the colony for the duration of the experiment although, surprisingly, they were the last to suffer mortality and colony death. As DAC3

began with the largest number of workers, they may have been better able to maintain a favourable brood temperature (Weidenmüller *et al*., 2002) to promote the emergence of the brood and maintain a higher number of live workers. It is also possible that foraging from this colony occurred outside the observation period. Considering the evidence presented in this study, our findings suggest that, in Birmingham, colonies initiated at the end of summer by bivoltine queens are unlikely to survive winter.

Field experiments: Simulating early terminating colonies (ETCs)

In this study, immature colonies established at the end of winter/early spring emergence were also able to produce workers for up to 3 weeks after being placed in the field – albeit in very low numbers. This again may have been as a result of the emergence of brood first initiated at favourable temperatures within the lab, before being placed in the field. However, after a snow event on the third day of the field experiment (22nd March 2013) followed by the death of all 4 queens (between 22nd March and 2nd April), colony collapse occurred quicker than in the previous field experiment initiated in October. March 2013 had a lower minimum and maximum temperatures than March 2012 and March 2014 (Figure 5.4; Met Office, 2012; 2013; 2014). Due to the immature nature of the ETCs, there were also fewer individuals to maintain a constant brood temperature (Weidenmüller *et al*., 2002) and to forage for pollen and nectar (Peat and Goulson, 2005). Temperatures recorded inside the colony were lower than those recorded for DAC colonies in October – December 2012 (Appendix B), with little evidence of colony heat generation and the maintenance of a constant temperature. Had this experiment been conducted in March 2012 or 2014, with milder temperatures (Figure 5.4), the

outcome may have been more favourable. However, the experiment could not be repeated due to time constraints.

Earlier emergence has been reported in many bee species as a result of climate change (Bartomeus *et al*., 2011; Skyrm *et al*., 2012) including in wild bees (Kudo and Ida, 2013). However, as evidenced in this study, with unusually low temperatures at the start of spring, extreme events can negatively impact the survival of colonies founded by early emerging queens. Extreme events are predicted to be more common as a result of climate change (Williams *et al*., 2015), which may hamper the survival of colonies established by early-emerging queens. Colony starvation and death can have a drastic impact on pollinator service provision and, in order to mitigate the effects of climate change on *B. t. audax*, further studies are needed to investigate the 'cues' that determine the entry to and exit from diapause. An investigation is needed, for example, to assess if climate change has reduced the critical photoperiod required for diapause, as it has in some species (Sgolastra *et al*., 2011).

Conclusion

Results of this study indicate that *B. t. audax* is unlikely to become bivoltine and establish successful winter active colonies as far north as Birmingham. There is no evidence of wild colonies persisting through winter months, and commercial colonies were unable to sustain themselves when placed in the field under autumn or early spring conditions. There may be a temperature-dependent physiological barrier to the advancement of winter-active bumblebees northwards, and this has been proposed for other bee species. For example, the large carpenter bee (*Xylocopa virginica* L.) (Hymenoptera: Apidae), native to North America, is constrained northwards by low temperature and westwards by precipitation (Skandalis *et al*., 2011). Other data within this thesis (Chapter 4) also suggest foraging activity is greatly constrained at temperatures below 5°C. Climate warming may, in future, facilitate the survival of winter active bumblebees northwards of London (as in Stelzer *et al*., 2010), however, this must be coupled with adequate nest sites and sufficient floral resources (Goulson, 2002; Goulson *et al*., 2008). Of greater concern is recent warmer autumn conditions followed by extreme winter conditions. Thus queens may be averting diapause and attempting to produce a second colony, but these colonies fail. This would have profound effects on bumble bee abundance and distribution.

CHAPTER 6

THE EFFECT OF THE NEONICOTINOID THIACLOPRID ON THE SURVIVAL AND THERMAL ACTIVITY THRESHOLDS OF *BOMBUS TERRESTRIS AUDAX* AND *B. T. DALMATINUS*

6.1. ABSTRACT

Pesticides are often present in the environment at levels insufficient to cause direct mortality. However, recent evidence suggests that sub-lethal concentrations of neonicotinoids can be detrimental to bee foraging behaviour with long-term impacts on colony viability. For the first time in any pollinator, I examine the effect of sub-lethal exposures to a neonicotinoid pesticide on thermal activity thresholds. When investigating lethal concentrations, the field-recommended concentration of thiacloprid (180ppm), recommended to be sprayed on domestic plants, resulted in survival rates of 53.3 ± 6.7% and 36.7 ± 8.0% for *B. t. audax* and *B. t. dalmatinus* respectively. There was no significant difference in survival between the subspecies over a range of thiacloprid concentrations. When investigating sub-lethal concentrations, exposure to 40ppm thiacloprid significantly increased the lower temperature threshold for coordinated movement (CTmin) in *B. t. audax* workers from 4.2 ± 0.54°C in controls, to 17.7 ± 0.56°C after 24h exposure ($p<0.05$). The temperature at which bumblebees recovered from chill coma and regained activity also increased significantly from 0.8 ± 0.2°C and 8.8 ± 0.5°C in controls to 9.7 ± 1.0°C and 12.5 ± 1.0°C respectively. This represents preliminary evidence that concentrations deemed safe for domestic pesticide applications can be lethal. In addition, there are clear sub-lethal effects that could significantly impact on

individual (and colony) performance. The wider implications posed by more potent neonicotinoids are discussed.

6.2. INTRODUCTION

Since their introduction in the early 1990s, neonicotinoids have become the most widely used class of insecticide in the world (Goulson, 2013). They are licenced to control pest species (Blacquière *et al*., 2012) in 140 agricultural crops and in many domestic horticultural products, generating $1 billion in sales in 2009 (Stoksad, 2013). Neonicotinoids are primarily applied as a seed coating (Whitehorn *et al*., 2012) and are translocated to all parts of the plant via phloem and xylem transport (Bromilow *et al*., 1990; van der Sluijs *et al*., 2013). As a result of their possible negative impact upon pollinators, a two year ban has been imposed on a number of neonicotinoids in Europe (clothianidin, imidacloprid and thiamethoxam; Stoksad, 2013). However, some neonicotinoids are still available for purchase and application in the UK, one such example is the neonicotinoid thiacloprid (Goulson, 2013; The Soil Association, 2013; Royal Horticultural Society, 2014a).

Thiacloprid is available to untrained members of the public in various foliar, horticultural sprays (Goulson, 2013; Royal Horticultural Society, 2014a). These are aimed at eliminating pests (such as: aphids, scales, mealybugs, leafhoppers, thrips, capsid bugs, small caterpillars, sawfly larvae and leaf beetles), and are approved for use on ornamental plants, fruits and vegetables (Royal Horticultural Society, 2014a). Gardens which have been sprayed with

neonicotinoids are likely to be visited by wild and managed pollinators (Lye *et al*., 2011; Defra, 2012; Goulson, 2013), given that gardens provide forage for pollinators throughout the year (Hunter and Hunter, 2008). Although thiacloprid lacks the level of toxicity of pesticides from the N-nitroguanidine group, Iwasa (2004) and Laurino *et al*. (2011) found evidence to suggest the possibility toxicity to pollinators. Being water soluble, they are absorbed by both roots and leaves and protect all areas of the plant from herbivory damage (Goulson, 2013). The unregulated spraying of neonicotinoids in gardens therefore presents a concern to pollinator health.

Environmental neonicotinoid levels have been found to overlap with those known to cause mortality (Goulson, 2013), hence the global decline of pollinators has been, in part, attributed to the ubiquitous use of neonicotinoids in crop management (Blacquière *et al*., 2012). The main neonicotinoids used in agriculture are imidacloprid, thiacloprid, thiamethoxam, clothianidin, dinotefuran, acetamiprid, nitenpyram and sulfoxaflor (Liu *et al*., 2008; Casida, 2010; Cutler *et al*., 2013; van der Sluijs *et al*., 2013). These can be partitioned into three groups; the N-nitroguanidines (imidacloprid, thiamethoxam, clothianidin and dinotefuran), nitromethylenes (nitenpyram) and N-cyanoamidines (acetamiprid and thiacloprid) (Jeschke *et al*., 2011; Goulson, 2013). The N-nitroguanidines are recognised to be more toxic to arthropods, with the addition of a nitro-containing group, as opposed to a cyano–group N-cyanoamidines (Iwasa *et al*., 2004; Laurino *et al*., 2011; Blacquière *et al*., 2012), as a result of the fast bio-transformation of the N-cyanoamidines (Brunet *et al*., 2005; Blacquière *et al*., 2012).

Neonicotinoids are a class of nicotinic acetylcholine receptor agonists (nAChRs) which are highly neurotoxic to insects (van der Sluijs *et al*., 2013). They mimic the acetylcholine neurotransmitter in the post-synaptic membrane of insect central nervous systems (Goulson, 2013) and bind with high affinity (van der Sluijs *et al*., 2013). In high concentrations, this results in a blockage of nAChRs resulting in paralysis and death (Tomizawa and Casida, 2003). At low concentrations, this promotes nervous stimulation (Matsuda *et al*., 2001), resulting in a variety of symptoms including trembling, a lack of coordination and hyperactivity (Blacquière *et al*., 2012).

Due to the systemic nature of neonicotinoids, their presence in the pollen and nectar of flowering crops has recently been highlighted as a potential threat to pollinators (Whitehorn *et al*., 2012). When used as a seed coating, for example, sunflowers treated with 0.7mg ^{14}C-imidacloprid, resulted in pollen and nectar levels of 3.9ppb and 1.9ppb respectively (Schmuck *et al*., 2001). When used as a foliar spray, however, levels are typically higher: after apple trees were sprayed with a foliar thiacloprid (0.2kgha^{-1}), the amount of thiacloprid in pollen after 1 and 6 days was 0.06 and 0.03ppm respectively (Blacquière *et al*., 2012). This pollen is often returned to the colony and fed to the developing brood, which may be affected differently to adult bees, providing an additional problematic factor to bee health (Godfray *et al*., 2014). Also, as neonicotinoids persist in soils (van der Sluijs *et al*., 2013), they can often be transferred to wildflowers in field margins (Whitehorn *et al*., 2012; Goulson, 2013). Krupke *et al*. (2012), for example, found neonicotinoid concentrations of up to 9 parts per million (ppm) on wild flowers growing near treated crops.

When assessing the impact of neonicotinoids on pollinators, initial studies focused on mortality and lethal concentrations (Suchail *et al*., 2000). However, in a meta-analysis of 13 studies looking at the effects of imidacloprid on honeybees, Cresswell (2011) found no significant effects. This suggests minimal environmental relevance of mortality studies. Focus was then directed to the sub-lethal effects of neonicotinoids, which, in honeybees, were examined in an extensive review by Desneux *et al*. (2007). Briefly, sub-lethal neonicotinoid effects on honeybees are reported to include impaired larval development, compromised immunological response, decreased fecundity, an alteration of sex ratio, impaired mobility, navigation, feeding behaviour and learning performance. In honeybees, Yang *et al*. (2012) also found impaired olfactory associative behaviour after 0.04nglarva^{-1} imidacloprid was administered. They also found a decrease in brood capping rates after imidacloprid concentration was increased from 24 to 8000nglarva^{-1}. In bumblebees, Whitehorn *et al*. (2012) reported an 85% reduction in queen production as a result of a field-realistic dose of neonicotinoids, compared to a control. Mommaerts *et al*. (2010) found that sub-lethal neonicotinoid concentrations negatively impacted foraging behaviour of workers and therefore hindered colony growth and survival. Colony size is particularly important in sub-optimal temperatures, where smaller colonies were found by Weidenmüller (2002) to be less able to maintain a constant temperature of between 30 and 32˚C (Heinrich, 1975) for brood development. It follows, therefore that smaller colonies are more likely to fail as a result of extreme environmental temperatures. The effects of pesticide exposure in combination with sub-optimal temperatures in any pollinator, however, have not been investigated.

The effect of compounding stressors on insect survival and behaviour has been investigated in several studies. Cross-tolerance is exhibited in a range of insect species between stressors such as desiccation and cold tolerance (Bayley *et al*., 2001; Ramløv and Lee, 2000) and anoxia and thermotolerance (Wu *et al*., 2002). There is also some evidence that exposure to toxins, such as heavy metals can enhance thermal tolerance (Holmstrup *et al*., 2010). There are very few studies available examining cross-tolerance of pesticide exposure and another stressor. In a study by Vidau *et al*. (2011), honeybees exposed to sublethal concentrations of thiacloprid ($5.1mgl^{-1}$, corresponding to $1/100^{th}$ of the LD_{50}) and infected by the parasite *Nosema ceranae* suffered increased mortality when compared to control samples. The only reference to pesticide cross tolerance (specifically thiacloprid) with temperature stress was in an unpublished MSc project. This study found $125mgkg^{-1}$ thiacloprid resulted in 90% average mortality in the springtail *Folsomia candida* Willem (Collembola: Isotomidae) at 15°C, but this decreased to 70% mortality at 7°C (Tesfamichael, 2011).

Due to the increasing evidence suggesting the negative impact of pesticides on bees, the European Commission imposed a two year ban, as of December 2013, on the planting of seeds treated with clothianidin, imidacloprid and thiamethoxam and the spraying of these insecticides on crops which are preferential to bees (Stoksad, 2013). However, a common domestic spray, containing thiacloprid (Provado Ultimate Bug Killer Concentrate) is available from a number of retailers (Table 1.1). Urban parks and gardens support a wide variety of beneficial insects (Hunter and Hunter, 2008), and have been recognised to contribute to the phenomenon of winter active bumblebees in the UK (Stelzer *et al*., 2010). The application of

domestic pesticides such as thiacloprid could be extremely detrimental to colonies of winter active bumblebees, given the reported sub-lethal effects of neonicotinoids (Mommaerts *et al*., 2010) and the high levels of thermal stress experienced by bivoltine bumblebees in the UK (Chapter 5). This is especially relevant in a domestic setting, because it is urban gardens that often provide forage for winter active bees (Stelzer *et al*., 2010).

In this study, I record the lethal concentrations of thiacloprid for *B. t. audax and B. t dalmatinus* as a result of oral consumption via nectar. I then investigated the effects of a sublethal thiacloprid concentration on activity thresholds (see Chapter 4) of *B. t. audax* specifically. Oral consumption has been identified as a route of exposure of bees to neonicotinoids in the wild (van der Sluijs *et al*., 2013) and it occurs when bees forage for the nectar of contaminated flowers. This represents the first ever study of how pesticide exposure could impact upon activity thresholds of a key pollinator, as well as emphasizing the risks of unregulated domestic pesticide application.

6.3. METHODS

Culture system

Mature colonies of *B. t. audax* and *B. t. dalmatinus* obtained from Biobest NV (Westerlo, Belgium), were maintained at 20ºC in constant darkness and manipulated under red illumination to minimise disturbance (Sadd, 2011). Nectar was available within the colony using a wick system connected to a reservoir of BioGluc nectar and pollen paste was available *ad libitum* (Biobest NV).

Determination of lethal and sub-lethal thiacloprid concentrations

This study used a commercially-available pesticide, 'Provado Ultimate Bug Killer Concentrate' (Bayer Garden©; Trade Name PROV ULTBUG CONC EYD SE9 12X400ML BOT GB), which contained $9gl^{-1}$ thiacloprid. Experiments were undertaken which aimed to identify thiacloprid concentrations which did not cause mortality in *B. t. audax* or *B. t. dalmatinus*. Using serial dilutions, a range of thiacloprid concentrations were mixed with nectar and fed to bees. They ranged from the undiluted product (6757 parts per million) through 4505, 2252, 180 and 90ppm, to concentrations which did not result in mortality (9 and 40ppm). The manufacturer's instructions specified 180ppm as the recommended concentration to be sprayed on to tomato plants of which bumblebees are a key pollinator. Bumblebees were exposed to thiacloprid concentrations (mixed with nectar) as follows. Workers were kept in 'feeding boxes' (as in Chapter 2) in groups of 5 and fed contaminated nectar and fresh pollen *ad libitum* for 24h. There were a total of 6 feeding boxes tested per

thiacloprid concentration, per subspecies. Survival was assessed after 24h, maintaining the experimental replicates, by assessing response to gentle manipulation (see previous chapters).

Long term survival after thiacloprid exposure

Worker bumblebees of both subspecies were exposed for 24h to 40ppm thiacloprid, which was known not to cause mortality from earlier experiments (Figure 6.1). They were then transferred to non-contaminated conditions and their survival was monitored, as previously described, for a period of 9 days. Totals of 15 *B. t. audax* and 15 *B. t. dalmatinus* workers were tested, in groups of 5 individuals. Controls with non-contaminated nectar were run simultaneously (n=15 per subspecies).

Activity thresholds after exposure to a sub-lethal concentration

Individuals of subspecies *B. t. audax* were fed nectar containing 0 (control), 0.009, 9, 20 or 40ppm thiacloprid for 24h before their activity thresholds were measured (see Section 4.3). Concentrations of 9 and 40 were selected as they did not result in mortality in earlier experiments, and concentrations of 0.009 and 20ppm represented serial dilutions and intermediate concentrations of thiacloprid respectively. Bumblebees were added in groups of 2, to an aluminium arena, programmed to cool at a rate of 0.1°Cmin^{-1}, and the temperatures at which they lost the ability for co-ordinated movement (CTmin) and entered a coma (chill coma) were recorded. The temperatures at which bumblebees recovered from chill coma (chill coma recovery) and regained co-ordinated movement (activity recovery) were also recorded

in a fresh set of individuals. The total number of individuals for which CTmin and chill coma were recorded was 15, 8, 11, 10 and 9 for pesticide concentrations 0 (control), 0.009, 9, 20 and 40ppm respectively. Due to limitations on the number of bees available for experimentation, chill coma recovery and activity recovery experiments were only undertaken on bumblebees exposed to 20ppm thiacloprid (for 24h). The total number of individuals for which chill coma recovery was tested was n=14 (and n=15 for controls) and activity recovery was n=9 (and n=9 for controls). Results were adjusted as a result of calibration as outlined in Chapter 4 (Section 4.4).

Statistical Analysis

A number of preliminary statistical tests were undertaken on the response variables to determine whether parametric or non-parametric testing could be employed. Normality was tested using Kolmogorov-Smirnov tests; equality of variance (homoscedasticity) was investigated using Levene's tests and time series were plotted to confirm the independence of samples. All response variables in this chapter were not normally distributed, but were homoscedastic and independent, therefore independent samples Kruskal Wallis tests with pairwise comparisons were undertaken using SPSS®. As multiple comparison tests in SPSS® include a correction for the familywise error rate, confidence interval bars on multiple comparison bar charts were adjusted using Bonferroni corrections (Figures 6.1, 6.3, 6.4 and 6.5). Chill coma recovery and activity recovery data were adjusted using the equations and methods described in Chapter 4.

6.4. RESULTS

Determination of lethal and sub-lethal thiacloprid concentrations

Survival decreased with increasing thiacloprid concentration (Figure 6.1). Exposure to 6757ppm for 24h resulted in 10 ± 4.47% survival for both subspecies, while concentration of 50ppm resulted in 96.67 ± 3.33% survival for *B. t. audax* and 93.33 ± 4.22% survival in *B. t. dalmatinus* (Figure 6.1). Concentrations of 40ppm and below were sub-lethal. These concentrations, plus intermediates, were used in all subsequent experiments. Survival was not considered significantly different between *B. t. audax* and *B. t. dalmatinus* subspecies at any concentration ($p>0.05$, $n=96$, $df=1$). Survival as a result of 6757ppm exposure was significantly different from 90, 50, 40 and 9ppm exposures ($p=0.015$, 0.002, 0.001 and 0.001 respectively, Kruskal Wallis test), and survival as a result of 4505ppm exposure was significantly lower than for samples exposed to 50, 40 and 9ppm thiacloprid ($p=0.19$, 0.007 and 0.007 respectively, Kruskal Wallis test).

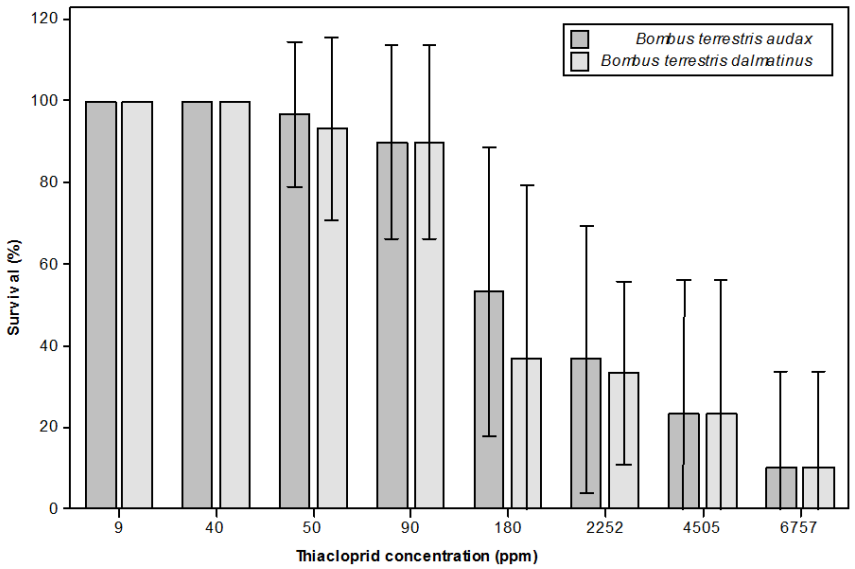
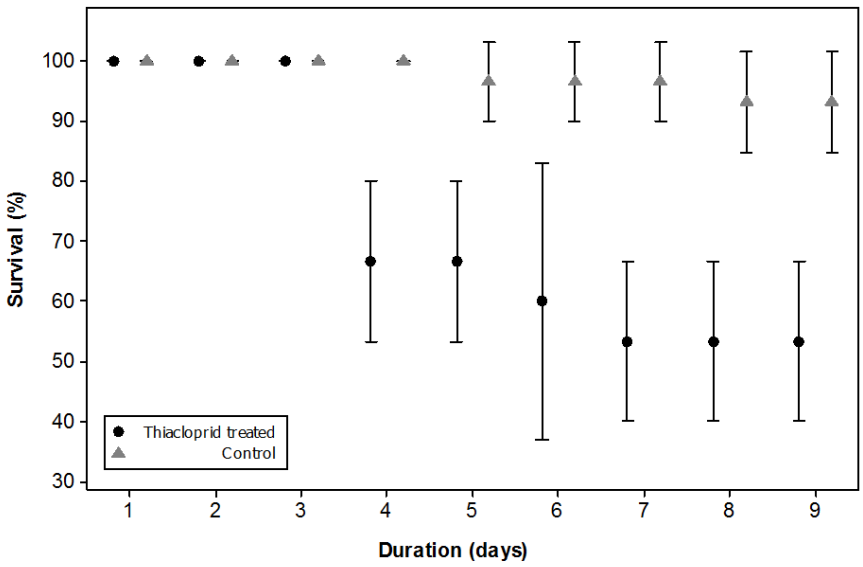


Figure 6.1 Mean survival (with Bonferroni-corrected 95% confidence interval bars) of *B. t. audax* and *B. t. dalmatinus* worker bumblebees after exposure to thiacloprid for 24h. Each bar represents 30 individuals.

Long term survival after thiacloprid exposure

Survival of both *B. t. audax* and *B. t. dalmatinus* workers exposed to 40ppm thiacloprid was 100% after 3 days, in line with controls (Figures 6.2a and b). Survival then decreased rapidly, and after a period of 9 days was 53.3 ± 6.7% in B. t *audax* and 40 ± 0% in *B. t. dalmatinus*, compared to controls (93.3 ± 4.22% for both *B. t. audax* and *B. t. dalmatinus*).

a)



b)

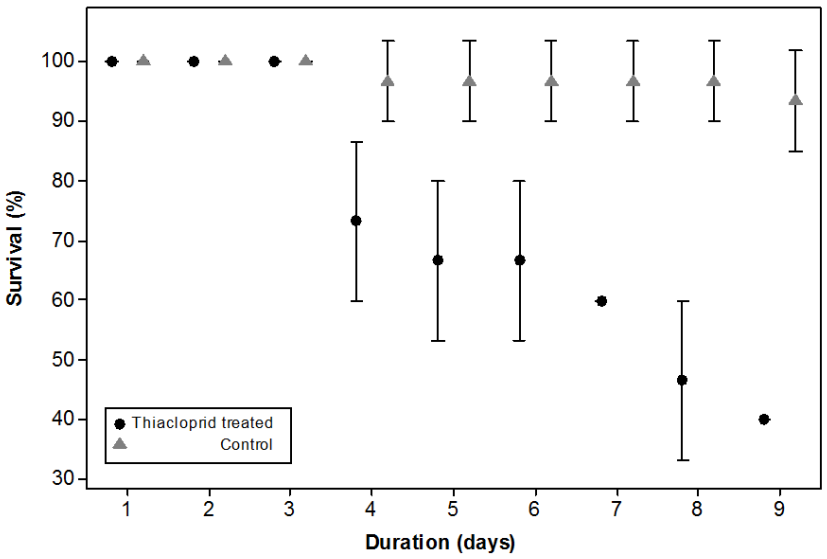


Figure 6.2 Mean daily survival (±2SE) of a) *B. t. audax* b) *B. t. dalmatinus* workers after an initial 24h exposure to 40ppm thiacloprid, versus control samples. 3 replicates of *n*=5 thiacloprid-treated bees and 6 replicates of *n*=5 bees for control samples.

Activity thresholds after exposure to a sub-lethal concentration

The CTmin of bumblebees (*B. t. audax*) to 0, 0.009, 9 and 20ppm thiacloprid were not significantly different ($p>0.05$) However, exposure to 40ppm increased the CTmin to 17.7 ± 0.56°C, compared to 4.2 ± 0.54°C recorded in control samples (Figure 6.3). This was significantly different from controls and all other concentrations ($p<0.05$ for all groups, $n=45$, $\chi^2=21.204$, $df=4$).

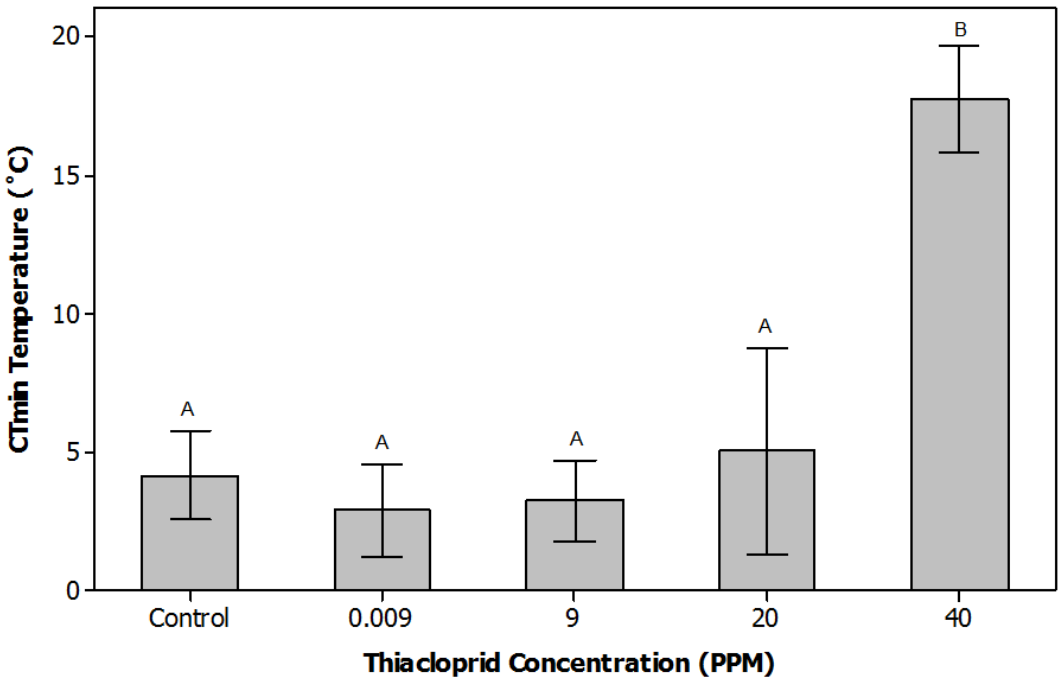


Figure 6.3 Critical thermal minima (CTmin) temperatures (with Bonferroni-corrected 95% confidence interval bars) of *B. t. audax* workers exposed to a range of thiacloprid concentrations for 24h immediately prior to experimentation, $n=15$, 8, 11, 10 and 9, for pesticide concentrations 0 (control), 0.009, 9, 20 and 40ppm respectively. Bars with differing letters represent a significant difference of $p<0.05$).

The highest chill coma temperature (-2.3 ± 0.9°C) was recorded for bees exposed to 9ppm thiacloprid (Figure 6.4). This was significantly different from controls (-5.1 ± 0.3°C), (p=0.01, n=53, χ^2=17.052, df=4). Differences between all other treatments were not significant and there was no consistent effect of thiacloprid exposure on chill coma temperature.

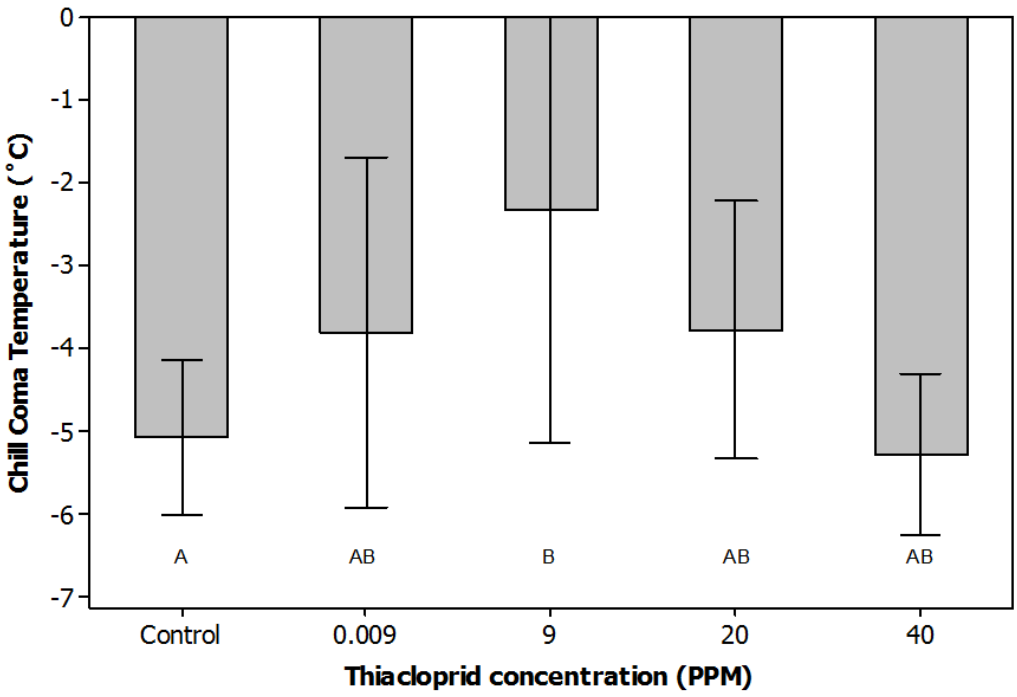


Figure 6.4 Chill coma temperatures (with Bonferroni-corrected 95% confidence interval bars) of *B. t. audax* workers exposed to a range of thiacloprid concentrations for 24h immediately prior to experimentation, n=15, 8, 11, 10 and 9, for pesticide concentrations 0 (control), 0.009, 9, 20 and 40ppm respectively. Bars with differing letters represent a significant difference of p<0.05).

The chill coma recovery and activity recovery were assessed for control and 20ppm thiacloprid exposures only, due to a shortage in the number of bees available for experimentation. Chill coma recovery occurred at 9.68 ± 1.0°C in exposed *B. t. audax* workers, and was significantly higher than that for control bees (0.8 ± 0.2°C, p<0.001, n=29,

U<0.001, *W*=120.00, *se*=22.907; Figure 6.5a). The activity recovery temperature for exposed bees (12.5 ± 1.0°C) was also significantly higher than for controls (8.8 ± 0.5°C; *p*<0.01, *n*=18, U=8, W=53, se=11.319; Figure 6.5b).

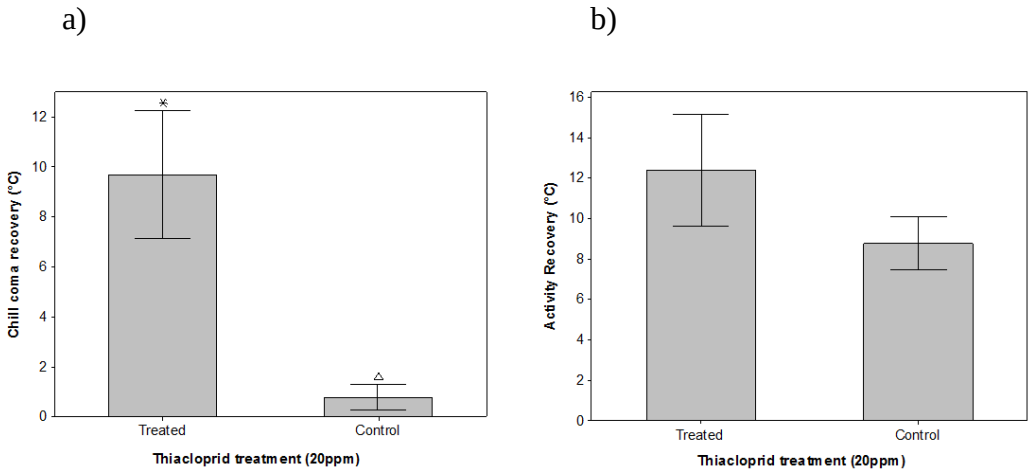


Figure 6.5 Mean (with Bonferroni-corrected 95% confidence interval bars) (a) chill coma recovery and (b) activity recovery temperatures, for untreated controls, and *B. t. audax* workers exposed to 20ppm thiacloprid ('treated') for 24h immediately before experimentation, *n*=14 and 15 for chill coma recovery and its control, *n*=9 and 9 for activity recovery and its control.

6.5. DISCUSSION

This study is the first to examine the impact of pesticide exposure on pollinator thermal activity thresholds. Results represent preliminary evidence that concentrations deemed safe for domestic pesticide applications can result in bumblebee death. Firstly, the field recommended concentration of thiacloprid in a domestic spray resulted in survival rates of just 53.3 ± 6.7% and 36.7 ± 8.0% for *B. t. audax* and *B. t. dalmatinus* respectively. For a product considered 'safe' for bees, which requires no training or usage licence (United States Environmental Protection Agency, 2003; Goulson, 2013; Bayer Crop Science, 2014; Royal

Horticultural Society, 2014a), this is a real concern. Sub-lethal thiacloprid concentrations, well below the levels recommended by manufacturer guidelines for the spraying of plants, significantly impacted on both CTmin and chill coma recovery, with workers losing coordinated movement at 17.7 ± 0.56°C when exposed to 40ppm thiacloprid for just 24 hours, compared to 4.2 ± 0.54°C for controls. Climate warming is likely to increase the chance of bumblebee queens averting diapause at temperate latitudes, resulting in winter active colonies (Stelzer *et al*., 2010). A greatly restricted thermal activity window would have devastating consequences on the ability to forage between autumn and spring resulting in total colony failure.

This study tested a broad spectrum of thiacloprid concentrations from highly concentrated (6757ppm), which may be sprayed on to plants as a result of user error, through field recommended (180ppm) to concentrations of 9ppm, which encompass the concentrations used to investigate the neonicotinoid toxicity in honeybees (as in Laurino *et al*., 2011). Predictably, survival rates were very low at the highest concentration of 6757ppm (10 ± 4.47%) and, interestingly, even the manufacturer-recommended concentration of 180ppm resulted in just 53.3 ± 6.7% survival for *B. t. audax* and 36.7 ± 8.0% for *B. t. dalmatinus*. This result appears to contradict claims that thiacloprid does not adversely affect bees, if used in accordance with the application guidelines (United States Environmental Protection Agency, 2003; Bayer Crop Science, 2014). Concentrations of 40 and 9ppm resulted in 100% survival in experiments based on assessments made 72 hours post treatment (Figure 6.1) therefore these

concentrations, and intermediates, were used to investigate sub-lethal effects on activity thresholds.

Bumblebees may be more sensitive to thiacloprid than honeybees, as Laurino *et al*. (2011) reported 100% honeybee survival following exposure to 144ppm thiacloprid for 72h. However, the Laurino *et al*. (2011) study may have benefitted from longer-term monitoring, as this chapter (Figures 6.2a and b) shows that a decline in survival may occur after the 72h monitoring period. When considering long term survival experiments, both *B. t. audax* and *B. t. dalmatinus* exhibited 100% survival after 3 days. This duration post-treatment for survival assessments is standard for many stress treatments (Hughes and Bale, 2009; Coombs and Bale, 2014; Everatt *et al*., 2014; Martinou *et al*., 2014) though it is interesting to note that bee survival did eventually decline significantly relative to controls 4 days post-treatment. After this point, survival in both subspecies decreased at a faster rate than controls, suggesting a delayed reaction to thiacloprid exposure. This highlights the shortfalls in relying on acute lethal toxicity as a measure of pesticide toxicity, as in European pesticide directives (91/414) and the Federal Insecticide, Fungicide and Rodenticide Act in the USA (Blacquière *et al*., 2012).

Not only do bumblebees appear more sensitive to neonicotinoids than honeybees, they may also come into increased contact with neonicotinoids in groundwater as a result of their subterranean nesting sites (European Food Safety Authority, 2013). The results of the current study are also consistent with reports that thiacloprid is considerably less toxic than

imidacloprid. For example, Mommearts *et al*. (2010) reported that a concentration of 200ppm imidacloprid was sufficient to cause 100% mortality in a bumblebee colony in a matter of hours, and the LD_{50} of imidacloprid in honeybees was 0.192ppm (Decourtye and Devillers, 2010).

The most dramatic effect noted in this study was on CTmin values, with a 13.5 ± 0.55°C increase relative to controls following exposure to 40ppm thiacloprid. This suggests thiacloprid has an impact on the ability to move in a coordinated manner, which may be the result of permanent damage to nerves that initiate the muscle action potentials necessary for coordinated movement, as described by MacMillan and Sinclair (2011). Neonicotinoids also result in a blockage to the nicotinergic neuronal pathway, controlling synaptic transmission and muscle action potentials (Liu *et al*., 2008). This is likely to compound the nerve damage accumulated as a result of low temperature exposure, resulting in a loss of coordinated movement at a normally tolerable temperature. There was no clear trend in thiacloprid effects on chill coma temperature, and the reasons for this are unclear. Chill coma is the result of an inability to maintain ion homeostasis (MacMillan and Sinclair, 2011) and based on the results of this study it seems that intermediate concentrations (9ppm) have the greatest effect on this process. Chill coma recovery is also thought to be controlled by neuromuscular function (MacMillan and Sinclair, 2011), so it is perhaps unsurprising that this parameter is also affected by thiacloprid.

Although the two year ban on clothianidin, imidacloprid and thiamethoxam imposed by the

European Commission is potentially a step towards an improvement in European bee health, this study highlights the potential dangers of other neonicotinoids currently considered as safe (United States Environmental Protection Agency, 2003; Goulson, 2013; Bayer Crop Science, 2014; Royal Horticultural Society, 2014a). It also presents data suggesting that foliar sprays available to unlicensed members of the public may cause damage to key pollinators. Exposures deemed sub-lethal can be directly lethal following longer term monitoring, or indirectly lethal as a result of reducing performance (*e.g.* the ability to move and forage). It would seem a much more detailed evaluation of the ecophysiological consequences of these products, is required, particularly in the context of combined pesticide and temperature stress. Additionally, future studies based on these data should determine the levels of pesticide found in bee tissues, to confirm the precise concentration the bee has ingested.

Although imidacloprid was not studied here, it has a higher toxicity (Tomizawa and Casida, 2003) and therefore has the potential to impact pollinators in concentrations as low as those found in seed coatings (Godfray *et al.*, 2014). There are alternative pesticides to neonicotinoids, namely pyrethroids and carbamates (UK Government, 2014). However, many pests are resistant to pyrethroids, and carbamates, such as Primicarb, are toxic to birds, mammals and aquatic organisms (UK Government, 2014). This study suggests a renewed investment in alternative crop protection strategies, such as those listed in Appendix 2. This study represents preliminary evidence that concentrations deemed safe for domestic pesticide applications can be lethal. In addition, there are clear sub-lethal effects that could significantly impact individual (and colony) performance.

CHAPTER 7
GENERAL DISCUSSION

Overview

Pollination is an essential yet increasingly vulnerable ecosystem service (Potts *et al*., 2010a). With the worldwide demand for food production increasing (Aizen *et al*., 2008), the need for pollinators has never been greater. Pollinators, however, are declining at an alarming rate (Goulson *et al*., 2008). The predominant agricultural pollinator in the world, the honeybee *Apis mellifera*, has suffered a large decline in recent decades, with a 59% loss of colonies between 1947 and 2005 in the USA, and a 25% decrease in central European colonies between 1985 and 2005 (van Engelsdorp *et al*., 2008; Potts *et al*., 2010a; Potts *et al*., 2010b). The global cost of a loss of pollinators could be catastrophic, with an estimated loss of between €190 and €310 billion (Gallai *et al*., 2009).

There are many reasons for the global decline of pollinators. The main drivers have been identified as climate change, the introduction of non-native species, pesticide use and habitat loss (Goulson *et al*., 2008; Potts *et al*., 2010a). Climate change has been responsible for the decoupling of plant–insect interactions, range shifts and extinctions (Parmesan, 2006). For example, in a meta-analysis performed by Parmesan and Yohe (2003), spring emergence was found to be advancing 2.3 days per decade, associated with a range shift of 6.1km towards the poles in birds, butterflies and alpine herbs. There has also been an increase in extreme events, with organisms subjected to extremes in cold, heat, drought and precipitation, with varying

frequency and duration (Rosenzweig *et al*., 2001). The introduction of non-native species has also been detrimental to pollinators, with non-native plants, pathogens and pollinators all causing declines. For example, the introduction of non-native pollinators, as reported by Ings *et al*. (2005a) has been reported to cause declines in UK-native bumblebees as a result of out-competition and genetic contamination as a result of hybridisation. Finally, pesticides are considered to be a driver of pollinator decline, with the ubiquitous use of neonicotinoids since their introduction in the 1990s (van der Sluijs *et al*., 2013). As a result of climate change, pesticide use is set to increase, given the predicted increase in the diversity and population size of pest species (Thomson *et al*., 2010). As a result of concerns raised by some members of the scientific community, the European Commission banned the use of three key pesticides as seed coatings in December 2013 (Stoksad, 2013). However, many neonicotinoids are available to the general public for unmonitored use, and have been released for general sale after a small number of toxicity tests on few pollinators (Bayer Crop Science, 2014; United States Environmental Protection Agency, 2003).

Although much attention has been focused on the decline of the honeybee, the importance of alternative pollinators should not be underestimated (Potts *et al*., 2010a). Due to improvements in mass rearing methods, bumblebees (such as the non-native subspecies *B. t. dalmatinus*) have been employed as commercial pollinators since 1988 (Inari *et al*., 2005) and are now the sole pollinators of crops such as tomatoes (*Lycopersicon esculentum*). In 2004, 40,000ha of greenhouse tomatoes worldwide were pollinated by bumblebees, with an estimated value of €12,000 million (Velthuis and van Doorn, 2006). Despite the presence of

the UK-native *B. t. audax*, the commercial importation *B. t. dalmatinus* has proceeded in large numbers, without regulation (Ings *et al*., 2005a). It is currently believed that *B. t. dalmatinus* has established in the UK, presenting issues of competitive displacement of native bumblebees (Ings *et al*., 2005b).

As a result of their economic importance, it is essential that we understand how the drivers of pollinator decline: climate change, non-native species, pesticide use and habitat loss (Goulson, 2008; Potts *et al*., 2010a) are impacting bumblebees in the UK (both the native *B. t. audax* and the non-native *B. t. dalmatinus*). As a result of data presented in this thesis, it can be said that the issues of climate change, non-native species and pesticide use (and their outcomes) are inextricably linked (Figure 7.1), and must be investigated in combination.

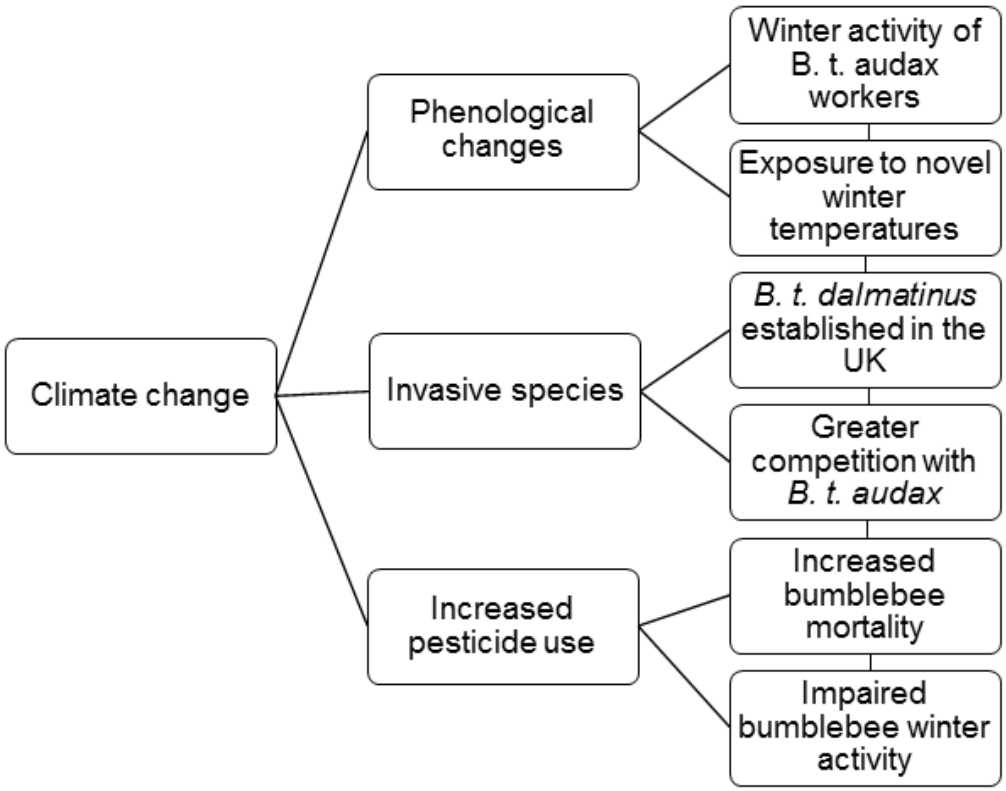


Figure 7.1. Climate change is driving phenological shifts, potential establishment of non-native species and increased pesticide use in *B. t. audax* and *B. t. dalmatinus*. The outcomes of each of these factors are also interlinked.

The interaction between climate driven phenological changes on the winter survival and performance of *B. t. audax* were assessed in Chapters 2, 3 and 4. These chapters also compared performance indices with the non-native *B. t. dalmatinus* to determine potential winners and losers under different climate scenarios. Chapter 5 then moved beyond the effects of temperature on individual bees, to consider the impact of winter activity on colony survival and performance. Finally, Chapter 6 considered the combined effects of thermal stress and pesticide exposure in the context of unregulated use of neonicotinoids in domestic and

urban/horticultural settings, which represent the most likely habitats to experience winter active bees.

Climate-driven changes in bumblebee phenology within the UK: winners and losers

The status of UK bumblebees at present is complex. There are several issues that may individually, or in combination, result in their decline (Goulson *et al*., 2008; Potts *et al*., 2010a). A primary issue is climate-driven changes in phenology of the native *B. t. audax*. Data from Stelzer *et al*. (2010) suggest bumblebees are altering their phenology in the south of the UK, and are becoming winter-active. This suggests that queens may be averting diapause and initiating a second colony at the end of summer. This second colony is then exposed to late autumn and potentially winter conditions before it reaches the point of being able to produce further queens. This scenario presents an interesting set of thermal challenges to bumblebee workers in particular, which have previously been confined to the summer months (Sladen, 1912). Another issue is that of the non-native bumblebee *B. t. dalmatinus*. Concerns have been raised that this subspecies has already become established in the UK (Ings *et al*., 2006), so its cold tolerance profile, relative to the native *B. t. audax*, may determine which subspecies has a competitive advantage under climate scenarios driving winter activity. Such temperature-dependent competition has been reported in the beetles *Nicrophorus arbicollis* and *N. defodiens* (Silphidae: Nicrophorus), with *N. defodiens* displacing *N. arbicollis* only at low temperatures (Wilson *et al*., 1984). This competition is heightened in times of patchy resources (Goulson, 2003), as is the case for bumblebees, which are declining as a result of habitat loss (Potts *et al*., 2010a).

Can bumblebees survive winter?

Data presented in this thesis suggest that, physiologically, both native and non-native bumblebees are ill-adapted to the thermal challenges presented by UK winters (Table 7.1; Figure 6.3). Short-term exposure to temperatures above -8°C result in up to 50% mortality in both subspecies, with the same mortality experienced as a result of one week at 0°C. Both subspecies also die upon freezing, at an identical temperature of -7.1°C (Table 7.1). Although underground *Bombus terrestris* colonies (Alford, 1975) may shield individuals from the most extreme temperatures, soil temperatures can be as low as 0°C for days at a time, as far south as Hertfordshire (UK Environmental Change Network, 2013). While *B. t. audax* survives for longer at 0°C that *B. t. dalmatinus* (Table 7.1), 50% mortality occurred after just 1 week at this temperature.

Table 7.1 A comparison of the key thermal thresholds reported in this thesis, including the ability of bumblebees to undergo rapid cold hardening (RCH) and acclimate (Acc) to a temperature of 10°C for 1 week.

	$LTemp_{50}$ (˚C)	**$LTime_{50}$ at 0 ˚C (days)**	**SCP (˚C)**	**CTmin (˚C)**	**Chill coma (˚C)**	**RCH?**	**Acc?**
B. t. audax	-7.8 ± 1.1	7.2 ± 1.1	-7.1 ± 0.2	4.2 ± 0.5	-3.1 ± 0.3	Y	N
B. t. dalmatinus	-6.1 ± 1.0	4.8 ± 1.1	-7.1 ± 0.2	9.6 ± 0.5	-1.8 ± 0.2	Y	Y

Particularly large colonies may be able to thermoregulate to maintain temperatures above ambient (Weidenmüller *et al*., 2002); however, individuals must be physiologically able to collect a large amount of forage to sustain the energy demands of such heat generation (Moret

and Schmidt-Hempel, 2000). The ability of a bumblebee to forage depends on its thermal activity thresholds (CTmin), as coordinated movement is necessary for foraging (Chapter 4). Results presented in this thesis suggest that bumblebees are unable to move in a coordinated manner (and thus forage) at temperatures below 4.2°C (for *B. t. audax*) and 9.6°C (*B. t. dalmatinus*). This suggests they would be unable to forage for much of winter, and thus be unable to provide sufficient forage to maintain favourable colony temperatures. Temperatures of between 30 and 32˚C (Heinrich, 1975) are required for successful development of the brood: if this temperature is not achieved, it can result in the emergence of smaller bees (Goulson and Sparrow, 2009). Smaller bees are poorer foragers because they have poorer visual acuity (Spaethe and Weidenmüller, 2002; Goulson and Sparrow, 2009) as well as collecting less pollen and nectar (Peat and Goulson, 2005). Additionally, at the end of the colony cycle, the queens that are produced have smaller fat reserves, which can negatively impact their winter survival (Beekman *et al*., 1998b; Goulson and Sparrow, 2009). Most importantly, smaller workers are less able to forage at lower temperatures (Peat *et al*., 2005), further hindering the thermoregulation of the colony. These issues may compound and result in colony collapse, adding to the global decline of pollinators currently being experienced (Potts *et al*., 2010a). This outcome is supported by the results presented in Chapter 5, where colonies were placed under field conditions to simulate two scenarios: 1) the aversion of diapause by queens and the initiation of a second, winter-active colony in autumn; 2) early termination of diapause by queens and the establishment of an early spring colony. In both experiments colonies failed to survive winter conditions, or produce new queens. This may have been, in part, due to the unusually wet and cold conditions experienced at the start and end of winter 2012/2013 respectively. When comparing transect walks undertaken in London

(as in Stelzer *et al*., 2010) with those undertaken in Birmingham (this study), the variation in temperature between the two locations must also be taken into account: with London experiencing higher winter temperatures (Met Office, 2014). In a commercial pollination scenario, research into UK-wide variation in bumblebee survival may therefore be beneficial.

In Chapter 4, temperature traces of cooled bumblebees showed little evidence of endothermy. This is in contrast to many studies which reported an inverse relationship between temperature and metabolic rate, for example, in the honeybee (*Apis mellifera*) (Harrison *et al*., 1996), the anthophorid bee *Centris pallida* (Roberts and Harrison, 1998) and the orchid bee *Euglossa imperialis* (Borrell and Medeiros, 2004). However, bumblebees in this study were tethered, in order to monitor temperature, and may have lacked the floral incentive to initiate heat generation. This is in agreement with Herrera (1995) and Woods *et al*. (2005), who found that tethered bees and bees requiring agitation to fly, failed to display endothermy.

Acclimation

Although laboratory measures of cold tolerance such as LTemp and LTime are useful, they do not account for the natural period of acclimatisation that occurs in the wild (Lagerspetz, 2006; Everatt *et al*., 2014). As temperatures slowly decrease, bumblebees have an opportunity to 'prepare' themselves for low temperatures and mitigate cold injury (Everatt *et al*., 2014; Lagerspetz, 2006). Essential to this preparation is the maintenance of cell membranes, namely the protection from breakage, phase transition and the correct functioning of membrane proteins (Koštál *et al*., 2013). Interestingly, only *B. t. dalmatinus* displayed the ability to

acclimate, with one week at 10°C resulting in a lower CTmin. This added ability brings the acclimated CTmin of *B. t. dalmatinus* in line with that of the native *B. t. audax.*

Will B. t. dalmatinus out-compete B. t. audax?

With a similar cold tolerance profile, an identical SCP and similar activity thresholds after acclimation, *B. t. dalmatinus* may be able to compete with *B. t. audax* in the UK climate, raising concerns for the future of *B. t. audax*. The non-native species is already phenologically predisposed to winter activity, with natural, polyvoltine life cycle (Rasmont *et al*., 2005), and it has been shown to have a superior foraging ability (Ings *et al*., 2005b).

Pesticides and climate change

Climate change is resulting in both a change in pesticide usage, and how pesticides impact the environment (Bloomfield *et al*., 2006). For example, higher mean temperatures and longer growing seasons are resulting in an increase in the variety and abundance of insect pests (Thomson *et al*., 2010). This has resulted in the increased application of pesticides to control pests and maintain crop yield (Rosenzweig *et al*., 2001). Climate change is also increasing winter rainfall and promoting crack formation in the soil: a scenario that promotes the runoff of pesticides and their penetration deep into soils (Bloomfield *et al*., 2006). Pesticides are then taken up in wildflowers and agricultural crops, on which pollinators feed, and filter into underground colonies in which pollinators, such as bumblebees, inhabit.

When approving pesticides for application, toxicity tests which monitor potential impacts on pollinators are usually only undertaken on honey bees (United States Environmental Protection Agency, 2003). However, this neglects the impacts upon other pollinators, and the sub-lethal effects of the pesticide. Results presented in this thesis highlight the importance of evaluating the sub-lethal effects of pesticides on organisms other than the honeybee, as concentrations that are considered 'safe' impaired the ability of bumblebees to remain active at even moderate temperatures. The threshold for coordinated movement was raised to such a high level (17.7 ± 0.56°C) that it would be predicted to impair foraging ability not only in winter, but in spring, autumn and even periods of summer in the UK (Figure 6.3). For example, colonies are used to pollinate crops in the open field, as far north as Scotland (Whitehorn *et al*., 2013), where temperatures would often be below the lower thresholds of movement, after pesticide exposure. The sub-lethal effects recorded by Gill and Raine (2014), also include a negative impact upon their pollen foraging ability and a change in floral preferences. Given that they were responsible for the pollination on 40,000ha of greenhouse tomatoes (*Lycopersicon esculentum*) in 2004, with an estimated value of €12,000 million, these findings have the potential to devastate the commercial pollination industry (Velthuis and van Doorn, 2006). While the pesticide tested here (thiacloprid) is not the most popularly used in an agricultural setting, it is important to note that the other neonicotinoids historically used in agriculture are even more toxic than thiacloprid. Thus, even very low exposures from seed coatings could impact bee activity thresholds.

Aside from agricultural pesticides, neonicotinoids are being sprayed in gardens by untrained members of the public. This study is the first to investigate the impacts of such domestic pesticide sprays on pollinators. Despite the fact that 93% of UK parents have used pesticides in the home (with the most common application in the garden; Grey *et al*., 2006), little consideration has been made of the impacts of their usage on beneficial organisms; the spraying of pesticides in gardens is particularly relevant, as gardens provide a rich source of forage for winter-active bumblebees (Stelzer *et al*., 2010), and indeed insects as a whole (Goddard *et al*., 2009). Urban gardens appear to be particularly important, as the density of bumblebee colonies found in urban gardens (36 nests ha^{-1}) was as high as those found in countryside hedgerows (20-37 nests ha^{-1}) (Osborne *et al*., 2008). If the forage collected by bumblebees in a garden is contaminated with pesticides, there can be two predicted outcomes. First, the bumblebee is unable to return to the colony, and dies in the field. The colony then suffers from a reduction in foragers and a decrease in the number of workers able to thermoregulate the nest (Spaethe and Weidenmüller, 2002). Second, the workers return to the colony with contaminated forage, which they feed to the developing brood. This may result in smaller workers, as was described by Gill and Raine (2014), which may impair the foraging ability of future generations.

Conclusions and recommendations

There are several strategies which can be employed to mitigate the negative effects of climate change, non-native species and pesticides. The recent restrictions to the importation of *B. t. dalmatinus* to the UK, and controls on the use and disposal of colonies (Natural England, 2013), are a promising start. However, Ings *et al*. (2005b) have suggested that *B. t. dalmatinus* may already have established in the UK, so these restrictions may have been introduced too late. Given the similarities in cold tolerance (Chapters 2 and 3), there is a strong possibility that competition will occur between *B. t. audax* and *B. t. dalmatinus*, as it has in other species (Wilson *et al*., 1984). The availability of suitable nest sites and floral resources are predicted to be the major sources of conflict. For example, in Japan, native bumblebees (*B. hypocrita sapporoensis* and *B. diversus tersatus*) are being out-competed by the non-native bumblebee, *Bombus terrestris* (Matsumura *et al*., 2004). This has resulted in a decline in the native species, due to the depletion of resources and a lack of nest sites (Matsumura *et al*., 2004).

Competition for nest sites can be reduced by conserving bumblebee nesting areas, which are currently in decline (Potts *et al*., 2010a). Bumblebees, for example, nest in linear countryside habitats, such as fence lines, hedgerows and woodland edges (Osborne *et al*., 2008), which are slowly being removed (Nicholls and Altieri, 2012). Hedgerows, for example, have added importance as they support native bees and rarer species (Moradin and Kremen, 2013). Attempts to encourage bumblebees to nest in artificial nesting boxes have often failed (Gaston *et al*., 2005); the conservation of natural habitat is therefore essential. In order to reduce competition between *B. t. audax* and *B. t. dalmatinus*, adequate floral resources must also be

available. This is especially important if *B. t. audax* is averting diapause and foraging in winter, when resources are sparse and competition is therefore heightened (Goulson, 2003). Urban parks and gardens provide a good source of winter forage (Stelzer *et al*., 2010), which should be enhanced if competition is to be avoided. Gardeners can be encouraged to make their garden more attractive to pollinators: for example, the 'Plants for Pollinators' initiative by the Royal Horticultural Society (2014b). Of added importance is the removal of neonicotinoids from domestic horticultural products. Results generated as part of this thesis will inform both pesticide production agencies and government legislators of the sub-lethal effects of domestic pesticides on bumblebees, and the detrimental impact they are predicted to have on both wild and managed pollinators.

Limitations and future work

Bumblebees used in this study were of commercial origin and were obtained from BioBest, Belgium from a stock culture of bumblebees. Due to the protected nature of the pollination industry, few details were able to be obtained regarding the rearing methods and the influence of issues such as inbreeding. Inbreeding is a key issue in honeybees, for example (Chapman *et al*., 2008). It has contributed to the large-scale infestation collapse of many North American colonies as a result of the *Varroa destructor* mite (Tentcheva *et al*., 2004; Potts *et al*., 2010), and has had vast implications for the pollination industry (van Engelsdorp *et al*., 2008). It is possible that inbred bumblebee colonies may be at risk of infection or infestation by parasites and pathogens, in the same way as honeybees. It is important, therefore, to determine the level of inbreeding in commercial bumblebee colonies and, to contextualise this study, how

representative commercial bumblebees are of wild bees. Future work, would involve a thorough comparison between wild bees and commercial bees, including cold tolerance and the effects of pesticides.

The issue of activity thresholds in wild bees was briefly examined in this thesis, with the recording of thoracic temperature in wild bees during cooling (Figure 4.5). Although results suggested that the commercial bumblebees were representative of wild bumblebees in this particular experiment, a more thorough analysis is needed. Results would inform the pollination industry as to the health of their stock, the need for measures to improve genetic diversity, and the efficiency of commercial bees as pollinators in sub-optimal conditions.

Finally, future studies should aim to elucidate the specific thermal and photoperiodic conditions that determine the initiation and termination of diapause. Through an understanding of these cues, we can better predict the impact of a changing climate on phenology.

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APPENDICES

APPENDIX A. Adapted from Brickell (1996)[i]. List of Autumn-Spring flowering plants at Winterbourne Botanical Gardens, Birmingham, UK. (Acc. No=Accession number).

Acc. No.	Family	Genus	Species	Region	Flower colour	Flowering period
11111202	Amaryllidaceae	*Narcissus*	*cyclamineus*	N.W.Portugal and N.W. Spain	Golden yellow	Early Spring
10111202	Buxaceae	*Sarcococca*	*confusa*	W. China	White	Winter
10511202	Buxaceae	*Sarcococca*	*hookeriana*	W. China	White	Winter
04690905	Buxaceae	*Sarcococca*	*ruscifolia*	W. and C. China	Cream/ white	Winter
09211202	Calycanthaceae	*Chimonanthus*	*praecox*	China	Yellow	Winter
11480207	Caprifoliaceae	*Lonicera*	*x purpusii 'Winter Beauty'*	N. Hemisphere	White	Winter and early spring
11960307	Caprifoliaceae	*Viburnum*	*tinus*	Mediterranean	White	Late winter and spring
10721202	Caprifoliaceae	*Viburnum*	*x bodnantense V.farreri x V. grandiflorum*	Europe/Garden origin	Rose/pink/ white	Over a long period – late autumn to spring
10571202	Cornaceae	*Cornus*	*mas*	Europe	Yellow	Late winter
15860908	Crassulaceae	*Pachyphytum*	*oviferum*	Mexico	Orange-red or Greenish red	Winter to spring

Acc. No.	Family	Genus	Species	Region	Flower colour	Flowering period
10181202	Ericaceae	*Erica*	*arborea*	Europe	Greyish white	Spring
07311202	Ericaceae	*Pieris*	*japonica*	East China, Taiwan, Japan	White	Late winter and spring
09381202	Hamamelidaceae	*Hamamelis*	*japonica*	Japan	Yellow	Mid and late winter
09391202	Hamamelidaceae	*Hamamelis*	*mollis*	China	Golden-yellow	Mid and late winter
07591202	Hamamelidaceae	*Hamamelis*	*x intermedia* (*H.japonica x H. mollis*)	E. Asia and N. America	Yellow, dark red orange	Early and midwinter
07441202	Iridaceae	*Crocus*	*chrysanthus*	Europe	Vary from cream to deep yellow	Late winter and early spring
03180205	Iridaceae	*Iris*	*unguicularis*	W. Greece, S. Turkey, W.Syria, Tunisia, Algeria	Pale lavender to deep violet with central band of yellow	Late winter and early spring
09671202	Colchicaceae/Liliaceae	*Colchicum*	*bornmuelleri*	Turkey	Pale to deep purple/pink flowers	Autumn

Acc. No.	Family	Genus	Species	Region	Flower colour	Flowering period
09641202	Colchicaceae/Liliaceae	*Colchicum*	*speciosum*	Arabian peninsula	Yellow anthers, white throats, pink/purple tepals	Autumn
09301202	Oleaceae	*Jasminum*	*nudiflorum*	W. China	Bright yellow	Winter and early spring
11650307	Oleaceae	*Osmanthus*	*delavayi*	W. China	White	Mid and late spring
07161202	Primulaceae	*Cyclamen*	*coum*	Wide dist *e.g.* Mediterranean, Iran, Somalia	Varying from white, pink carmine-red	Winter or early spring
12000307	Primulaceae	*Cyclamen*	*hederifolium*	Mediterranean	Pink	Mid and late autumn
09221202	Ranunculaceae	*Clematis*	*cirrhosa*	Europe	Cream	Late winter And early spring
22831112	Ranunculaceae	*Helleborus*	*lividus*	Majorca	Cream/green and purple	Midwinter to Early spring

Acc. No.	Family	Genus	Species	Region	Flower colour	Flowering period
02810105	Ranunculaceae	*Helleborus*	*niger*	Germany, Austria, Switzerland, Slovenia	White, with green/white centres	Early winter to Early spring
02800105	Ranunculaceae	*Helleborus*	*x hybridus*	Germany, Austria, Switzerland, Slovenia	White, purple, yellow, green and pink	Midwinter to mid spring

APPENDIX B

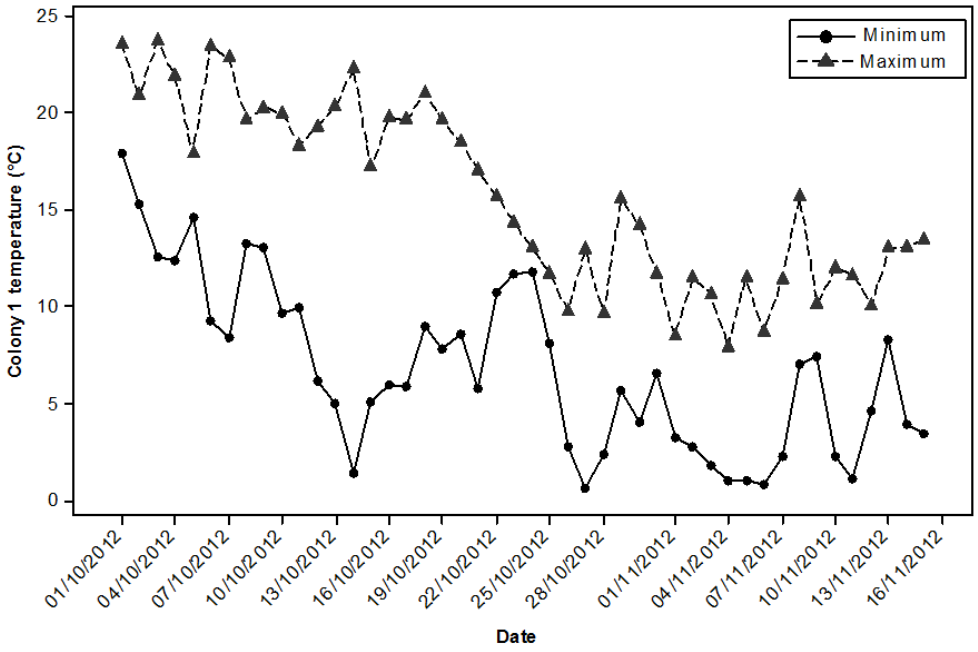

Figure B.1. Minimum and Maximum temperatures inside a commercial *Bombus terrestris audax* colony, located at Winterbourne Botanical Gardens, Birmingham, UK (Diapause Averting Colony 1, Chapter 5).

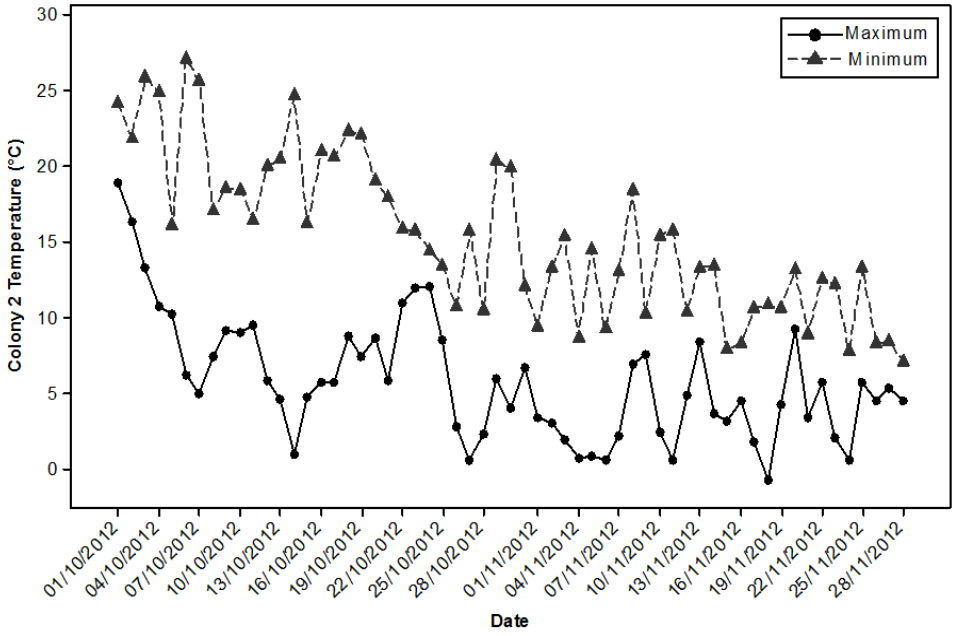

Figure B.2. Minimum and Maximum temperatures inside a commercial *Bombus terrestris audax* colony, located at Winterbourne Botanical Gardens, Birmingham, UK (Diapause Averting Colony 2, Chapter 5).

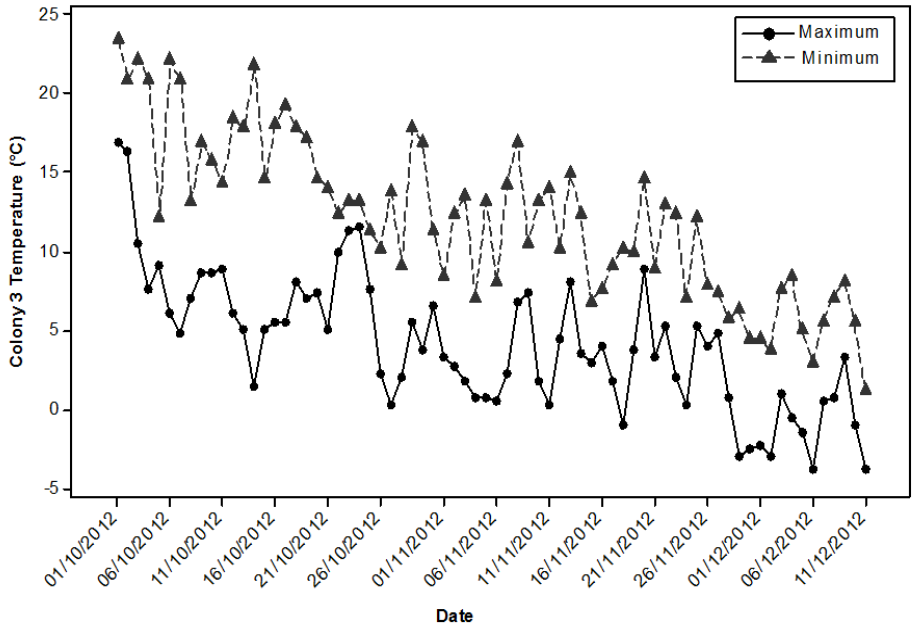

Figure B.3.Minimum and Maximum temperatures inside a commercial *Bombus terrestris audax* colony, located at Winterbourne Botanical Gardens, Birmingham, UK (Diapause Averting Colony 3, Chapter 5).

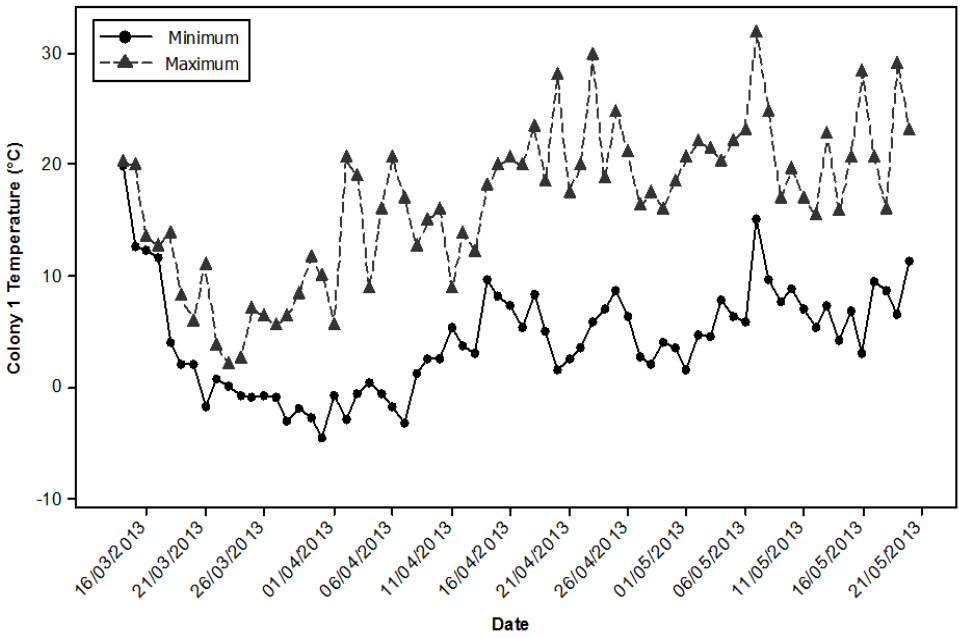

Figure B.4. Minimum and Maximum temperatures inside a commercial *Bombus terrestris audax* colony, located at Winterbourne Botanical Gardens, Birmingham, UK (Early Termination Colony 1, Chapter 5).

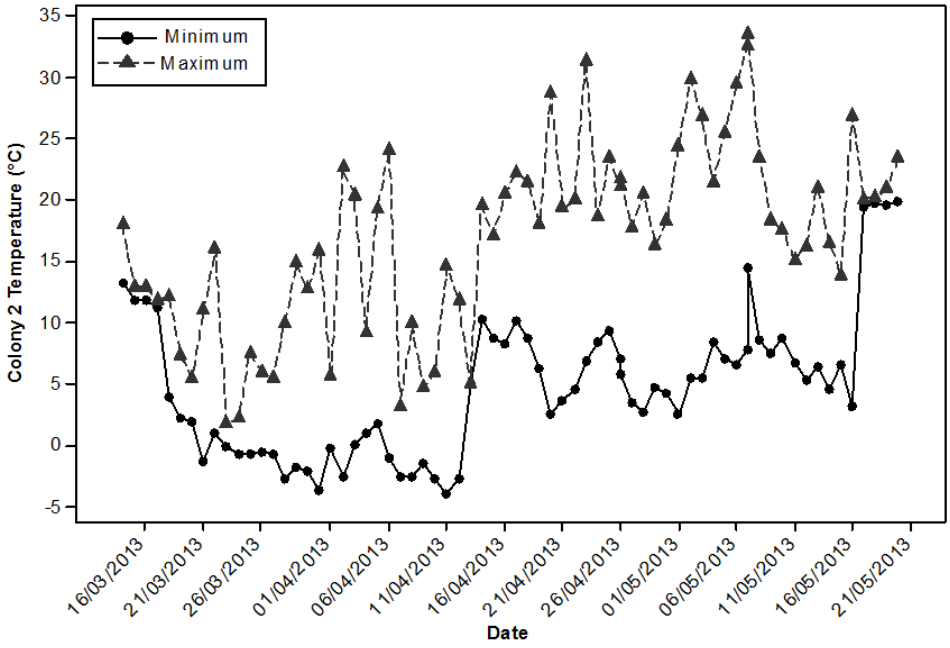

Figure B.5. Minimum and Maximum temperatures inside a commercial *Bombus terrestris audax* colony, located at Winterbourne Botanical Gardens, Birmingham, UK (Early Termination Colony 2, Chapter 5).

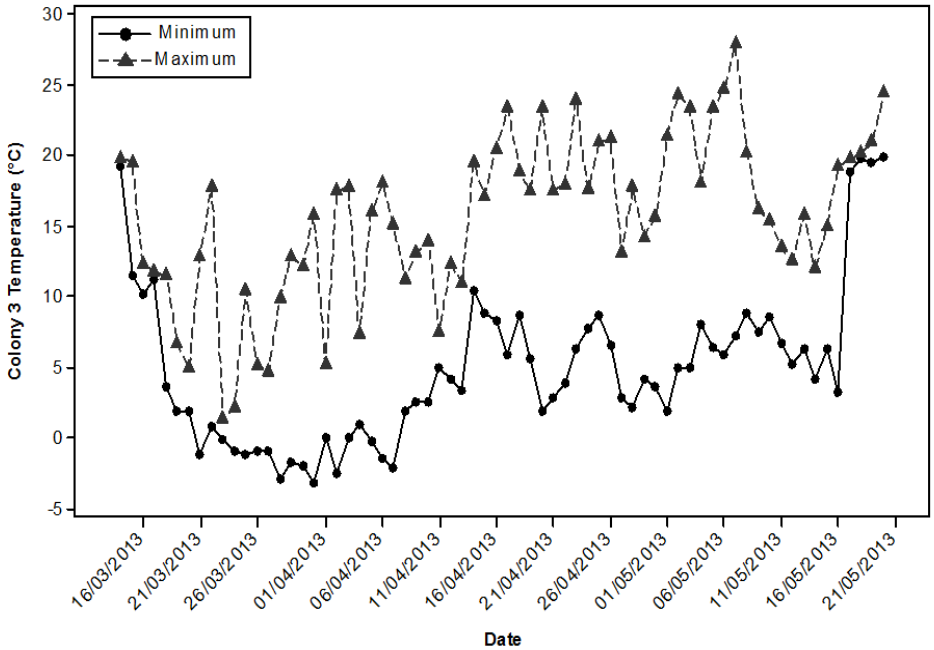

Figure B.6. Minimum and Maximum temperatures inside a commercial *Bombus terrestris audax* colony, located at Winterbourne Botanical Gardens, Birmingham, UK (Early Termination Colony 3, Chapter 5).

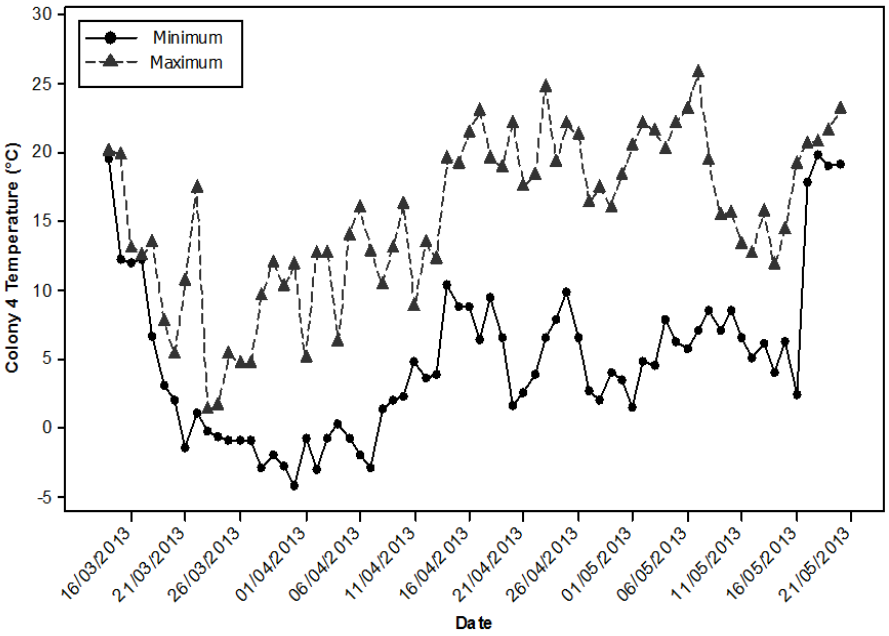

Figure B.7. Minimum and Maximum temperatures inside a commercial *Bombus terrestris audax* colony, located at Winterbourne Botanical Gardens, Birmingham, UK (Early Termination Colony 4, Chapter 5).

APPENDIX C.

Reproduced from Advisory Committee on Pesticides (2003)[ii]. Commercial and development status of alternative methods of control in the UK. Categories of development are 'commercial' – which means currently used, 'near market' which means either in the regulatory process or close to it and 'potential for future use' which means some way off commercial use. This is not an exhaustive list but is indicative of key alternative appr oaches.

Control method	Now used in the UK?	State of development	Potential future use
Pheromones	Yes. Product 'Exosex' approved on 13/09/04.	Commercial	High potential for 'attract and kill' in top fruit and field horticulture (*e.g.* codling moth, flour diamond back moth). Also high potential for mating disrupters.
Classical biological control	Yes. In glasshouses.	Commercial	Small – medium.
Entomopathogenic fungi	Yes. *Verticillium lecanii*.	Commercial	High in specific circumstances. *Beauvaria bassiana* pilot project – near to market.
Mycoherbicides	No.	Potential for future use.	Small.

Antagonistic fungi	Yes. *Peniophera* (*Phlebiopsis*) *gigantia*.	Commercial	Medium.
Microbial (virus) products	Yes. *Granulosis virus*.	Commercial	High in top fruit *e.g.* for control of codling moth.
Antifeedants/eating deterrants	No.	Potential for future use.	Small.
Plant extracts	Yes. Citronella. Garlic crude extract – sold as barrier.	Some near market, some commercial.	A range of plant extracts are fully approved, especially as cat and dog repellents. Also pyrethrins and rotenone as insecticides. Milsana (extract from giant knotweed) is marketed in Germany for powdery mildew on tomato; potential for use in UK.
Commodity chemicals	Yes.	Commercial	Wide uses in agriculture *e.g.* sulphuric acid, formalin, carbon dioxide.
Modified atmospheres	Yes.	Commercial	Used in stored products.

Physical and mechanical control	Yes. Many.	Commercial	High potential in high value crops.
Crop breeding	Yes. Many.	Commercial	High – but it is only one breeding objective. Genetic modification is an unknown technology.
Rotation	Yes.	Commercial	Specialist cropping has reduced need for rotation to date – could become more important in different economic situations.
Natural predator management	Yes. Field margins, beetle banks *etc*.	Commercial	High potential to increase its use, but efficacy is variable.
Forecasting	Yes. Many monitoring systems.	Some commercial and some near market.	High for some pests. Not universally applicable.

[i] Brickell. C. (1996) A-Z Encyclopedia of Garden Plants. *The Royal Horticultural Society*, Dorling Kindersley Ltd, London.

[ii] Advisory Committee on Pesticides (2003) Final Report of the Sub-Group of the Advisory Committee on Pesticides on Alternatives to Conventional Pest Control Techniques in the UK: A Scoping Study of the Potential for Their Wider Use. *Advisory Committee on Pesticides*, York, UK.