THE PHYSIOLOGY AND ECOLOGY OF DIAPAUSE UNDER PRESENT AND FUTURE CLIMATE CONDITIONS IN THE BLOW FLY, *CALLIPHORA VICINA*

By

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Abstract

Virtually all temperate insects overwinter in diapause, a pre-emptive response to adverse environmental conditions and for many species a pre-requisite of winter survival. Increased global temperatures have the potential to disrupt the induction and maintenance of diapause.

In the first part of this thesis, a four year phenological study of the blow fly, *Calliphora vicina*, identifies that diapause is already being delayed due to high temperatures experienced by larvae within the soil layer. Laboratory studies identified that non-diapause life stages are capable of heightening cold tolerance through a rapid cold hardening ability, and winter acclimated adults maintain locomotion at lower temperatures than summer acclimated adults. A previously unrecognised threat, however, is that higher adult temperatures have the transgenerational effect of reducing the cold tolerance of diapausing progeny.

In the second part of this thesis, the relationship between diapause and cold hardiness was investigated. The amino acid, alanine, was up-regulated as part of the diapause programme. Non-diapause larvae developed on an alanine augmented-diet expressed cold tolerance phenotypes similar to those of their diapausing counterparts. This adds to a growing body of evidence to suggest that amino acids have a direct role in insect cold tolerance.

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List of publications

Chapter 2

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Declaration of Author's contribution

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Paul Coleman (PCC), Scott Hayward (SH) and Jeff Bale (JB)

Chapter 1: Entirely my own work

Chapter 2: PC designed the experiment, with input from SH. PC collected the data, analysed the data and wrote the chapter. SH assisted with the discussion and analysis of data and SH and JB assisted with the writing of the manuscript.

Chapter 3: PC designed the experiment, with input from SH. PC collected the data, analysed the data and wrote the chapter. SH assisted with the discussion and analysis of data and SH has assisted with writing of the manuscript.

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Chapter 8: Entirely my own work

Abbreviations

AFPs – Antifreeze proteins

CDL – Critical day length

CT_{min} – Critical thermal minima

CT_{max} – Critical thermal maxima

d – day

D15 – Diapause larvae produced from adults acclimated to 15°C

D20 – Diapause larvae produced from adults acclimated to 20°C

DT – Discriminating treatment

DS – Diapause selected strain

DS15 – Diapause selected strain from adults at 15°C

DS20 – Diapause selected strain from adults at 20°C

h – hour

HSPs – Heat shock proteins

INAs – Ice nucleating agents

LD – Light:Day length

LT – Lethal time

ND15 – Non-diapause larvae produced from adults acclimated to 15°C

ND20 – Non-diapause larvae produced from adults acclimated to 20°C

NDS – Non-diapause selected strain

NDS20 – Non-diapause selected strain from adults at 15°C

NDS15 – Non-diapause selected strain from adults at 20°C

NMR – Nuclear magnetic resonance

PE – Protein excluded

PF – Protein fed

RCH – Rapid cold hardening

SCP – Supercooling point

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CHAPTER 1

GENERAL INTRODUCTION

1.1 Insect diversity

Fossil evidence suggests that insects were among the first animals to colonise the land around 400 million years ago (Labandeira and Sepkoski, 1993). Most modern insect orders have been in existence for over 250 million years (Grimaldi and Engel, 2005) and the oldest extant terrestrial species, discovered in 2008, is believed to be the *Martialis heureka* ant at 120 million years old (Rabeling et al., 2008). Since their early appearance in life's history, the Insecta have successfully colonised almost every environment on the planet. Sea skaters, *Halobates* spp, are found in the open ocean (Cheng, 1985), the midge, *Belgica antarctica*, is the largest purely terrestrial animal in the Antarctic (at 2-6 mm) (Lee and Baust, 1987) and thermophilic ants, such as *Melophorus bagoti*, are capable of tolerating temperatures approaching 60°C in the Australian desert (Christian and Morton, 1992).

There are an estimated one million insect species (Mayhew, 2007), with life spans ranging from minutes (Sweeney and Vannote, 1982) to decades (Thorne et al., 2002) and sizes from micrometres (Huber and Beardsley, 2000) to tens of centimetres (Parsons, 1986). Critical to their success is their ability to cope with a wide range of environmental conditions. At temperate latitudes, this involves highly specialised life-history strategies to survive periods of prolonged cold, limited food availability and other environmental stressors associated with the winter months (Denlinger, 2002). Fundamental to winter survival, for the majority of temperate insects, is a period of winter dormancy termed diapause, a genetically programmed, pre-emptive response to adverse environmental conditions (Denlinger, 2002). Traditionally viewed as a halt in development, diapause is now recognised as a highly adapted developmental pathway associated with complex physiological and biochemical changes (Saunders and Hayward, 1998; Hahn and Denlinger, 2007, 2011). For many temperate species this adaptive dormancy is a pre-requisite of winter survival, which has evolved to synchronise development with favourable environmental conditions (Hodkova and Hodek, 2004).

Given their diversity and highly adaptive life stages, it could be assumed that the Insecta will be resilient to current rates of climate change. Nonetheless, negative insect responses to climate change, such as a loss of synchrony with available resources, have already been observed (Sparks and Carey, 1995; Roy and Sparks, 2000; Parmesan and Yohe, 2003; Gordo and Sanz, 2005; Forrest and Miller-Rushing, 2010; Peñuelas et al., 2013). Understanding how insects will respond to climate change is of particular importance, because they play fundamental roles in key ecosystem services, such as pollination and nutrient recycling (Losey and Vaughan, 2006). They also represent major pests in agriculture (Zhang et al., 2007) and vectors of disease (Manachini et al., 2013; Oliveira et al., 2013; Shaw et al., 2013). Despite the recognised importance of insects, very little is known about how overwintering life stages will be affected by continued climate change (but see Bale and Hayward, 2010).

1.2 Thermal limits of survival

Every ecosystem on the planet will be affected in some way by climate change (Walther et al., 2002; Parmesan and Yohe, 2003; Hickling et al., 2006; Bellard et al., 2012). How species respond will in part be determined by the proximity of environmental temperatures experienced to the thermal limits of survival (Chown et al., 2010; Franks and Hoffmann, 2012; Hoffmann et al., 2013). All life on Earth, from unicellular protozoans, to the most complex multicellular organisms, require temperatures to remain within a relatively narrow thermal range. At either end of this thermal range are the upper and lower physiological limits of survival (Peck et al., 2009; Chown et al., 2010; Macmillan and Sinclair, 2011; Hoffmann et al., 2013). The upper thermal threshold is a result of a positive correlation that exists between temperature and oxygen demand (Pörtner, 2002). As temperatures increase so too does the demand for oxygen. Eventually demand will outweigh availability and if temperatures are not reversed the individual will succumb to mortality. At low temperatures oxygen supply also becomes limiting, due to the reduced ability of mitochondria to absorb

oxygen (Pörtner, 2002). Again, if temperatures are not reversed, the individual will reach the lower developmental threshold and eventually experience mortality.

In addition to upper and lower development thresholds, there is also an optimum developmental temperature (T_{opt}); a point at which metabolic activity, growth and reproduction are best suited (Huey and Stevenson, 1979; Jobling, 1981). Humans (*Homo sapiens*) have an internal T_{opt} of 36.8°C and upper and lower thermal thresholds 5°C either side of this temperature. This is a relatively narrow thermal range considering bacteria, such as *Escherichia coli*, have a thermal range of -18 to 49°C (Ehrlich et al., 1970). Humans, like all endotherms, are resilient to sudden temperature fluctuations, due to an ability to maintain constant homeostasis. This is in contrast to ectotherms, such as reptiles and arthropods, which are unable to maintain constant homeostasis, and instead rely on environmental temperatures remaining within their thermal threshold of survival (Davidson, 1944; Sunday et al., 2011). While relying on environmental conditions to control temperature may seem disadvantageous, ectotherms are able to tolerate a wider range of temperatures (i.e. a thermal breadth), due to a diverse array of high and low temperature tolerance mechanisms (Hutchison and Maness, 1979; Storey, 1997; Ramløv, 2000; Denlinger and Lee, 2010; Sunday et al., 2011). The thermal ranges of a hypothetical ectotherm and endotherm are summarised in Figure 1.1 as a relative fitness curve.

Interest in the upper and lower thresholds of survival has resurged in recent decades, as attempts are made to predict how species, from a range of taxonomic groups, are likely to respond to continued climate change (Peck et al., 2009; Macmillan and Sinclair, 2011; Hoffmann et al., 2013). The close association between temperature and relative fitness makes all life on Earth vulnerable to the effects of climate change. Indeed, biological responses to human-induced climate change have already been recorded.

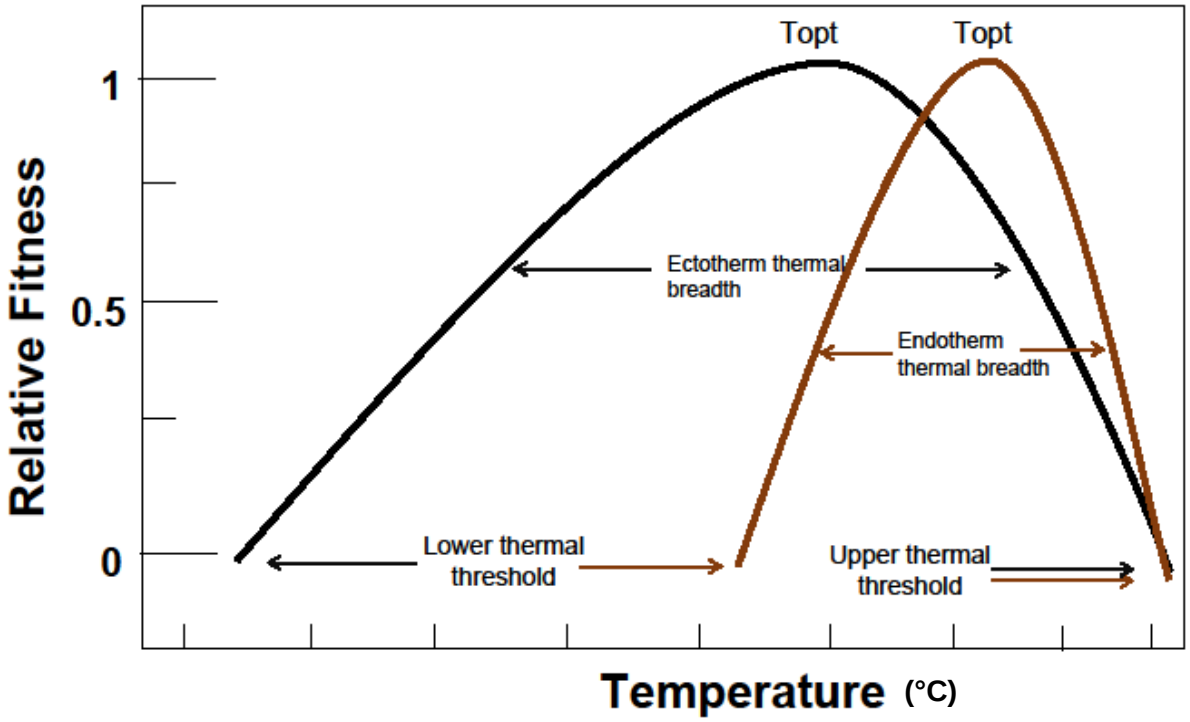

Figure 1.1 Relative fitness as a function of increasing temperature for a hypothetical endotherm (brown line) and a hypothetical ectotherm (black line). T_{opt} is the optimal developmental threshold and upper and lower thermal thresholds are also labelled Adapted from Huey & Stevenson 1979.

1.3 Responses to climate change

Elevated global temperatures have been identified as the main factor causing changes in the timing of biological events, altering multiple species distribution and modifying trophic interactions (Sparks and Carey, 1995; Roy and Sparks, 2000; Parmesan and Yohe, 2003; Gordo and Sanz, 2005; Forrest and Miller-Rushing, 2010; Peñuelas et al., 2013). Increased pest outbreaks (Volney and Fleming, 2000; Ladanyi and Horvath, 2010), species loss (Stork, 2009) and phenological shifts (Roy and Sparks, 2000) have all been attributed to climate change. Biological responses to climate change have been observed around the globe (Penuelas et al., 2002; Walther et al., 2002; Hoffmann and Sgrò, 2011). Leaf emergence on trees in the Mediterranean has advanced by 16 days over 50 years (Penuelas et al., 2002); average egg laying onset in UK birds has advanced by nine days over 25 years (Crick et al., 1997), and breeding in Canadian red squirrels, *Tamiasciurus hudsonicus*, has advanced by

18 days over 10 years (Réale et al., 2003). Similar advancements in yearly events have been documented around the globe, and a meta-analysis by Parmesan and Yohe (2003) concluded that on average, spring events are advancing by 2.3 days per decade in response to increasing temperatures. Insects, and all ectotherms, are particularly vulnerable to the effects of climate change (Bale et al., 2002; Bale and Hayward, 2010). While no insect extinctions have yet been attributed to climate change, Hoffman et al. (2013) suggest that they are inevitable as global warming continues. One of the most noticeable responses to elevated temperatures are pole-ward range expansions of species. For example, the Small Skipper Butterfly, *Atolopedes campestris* (Crozier, 2003) and Southern Green Stink Bug, *Nezara viridula* have both colonised regions where winter temperatures were previously below thermal development thresholds (Crozier, 2003; Tougou et al., 2009; Musolin, 2012). The range expansion of *N. viridula*, a prolific crop pest, is economically damaging for legume farmers throughout Asia, Africa and the Americas. Indeed, range expansions have been observed for a number of insect pest species, such as the green spruce aphid *Elatobium abietnum* (Westgarthsmith et al., 2007). Range expansions represent just one of many possible responses to climate change.

Deutsch et al. (2008) proposed that higher temperatures in the tropics, where temperatures are already approaching upper thermal thresholds, may be disadvantageous for tropical insects. The alleviation of cold stress, and increased frequency of T_{opt} conditions, may prove beneficial for temperate insects. This theory is based on the assumption that the alleviation of cold stress is the only response to climate change at high latitudes. In reality responses are likely to be more complex and species-specific, with negative responses to climate change already reported in some temperate insects such as the solitary bee, *Osmia lignaria* (Bale and Hayward, 2010).

There is mounting evidence to suggest that overwintering life stages are particularly vulnerable to the effects of climate change (Bale and Hayward, 2010). Warmer winters have been associated with heightened disease outbreaks and the rapid depletion of metabolic

reserves (Sgolastra et al., 2011; Radchuk et al., 2013). The emergence of active life stages before favourable environmental conditions have returned (Hahn and Denlinger, 2011) and the decoupling of photoperiod and temperature cues used by many insects to prepare for winter have also been reported (Bale and Hayward, 2010). The response of overwintering life stages to climate change will be the main focus of this thesis.

1.4 The biology of overwintering insects

For temperate insects winter is a time of low ambient temperatures, freezing conditions and limited nutrient availability (Tauber et al., 1986). Surviving this period of environmental stress is achieved through a combination of behavioural and physiological adaptations (Koštál, 2006; Hahn and Denlinger, 2011). For example, the Monarch butterfly, *Danaus plexippus*, avoids the winter in North America by migrating over multiple generations, to Central America (Urquhart and Urquhart, 1978). Another migratory insect is the hoverfly, *Syrphus ribesii*, which is a partial migrant. A proportion of the population migrate from the UK to the Mediterranean, while other members of the same population remain *in-situ* (Gilbert, 1984; Hart and Bale, 1998). The stationary proportion of the population overwinter in sycamore leaf litter (*Platanus* sp.) where bio chemical adaptations assist in winter survival (Gilbert, 1984; Hart and Bale, 1998). For the overwhelming majority of temperate insects the winter period is spent *in-situ* (Tauber et al., 1986; Mousseau and Dingle, 1991; Denlinger, 2002).

In-situ responses can be divided into two strategies: quiescence and diapause (Tauber et al., 1986). Quiescence is an immediate, aseasonal, response to environmental stress. It is induced by changes in temperature, moisture, or food availability. Quiescence can occur at any stage of the life cycle and development is quickly resumed once favourable conditions have returned – examples include many polar invertebrates, such as the Collembola species, *Cryptopygys sverdrupi* and *Gomphiocephalus hodgsoni* and the Acari, *Maudheimia wilsoni*, all of which have the capacity to overwinter at any stage of development (Convey, 1996). In contrast, diapause is a pre-emptive response to seasonal periods of environmental stress

(Denlinger, 1991). It is not induced directly by low winter temperatures, but is programmed by 'token' stimuli, most often photoperiod, that precede winter. Diapause is not terminated as soon as conditions become favourable, but must go through an ordered series of termination processes before development is resumed. For many temperate species, diapause is a pre-requisite of winter survival (Denlinger, 2002; Hodkova and Hodek, 2004).

Diapause can be either obligatory or facultative (Tauber et al., 1986). Obligatory diapause is a genetically determined response that occurs during a specific stage of the life cycle, irrespective of prevailing environmental conditions. Examples include the Garden tiger moth, *Arctica caja* (Lees, 1955), the Cecropia Moth *Hyalophora cecropia* (Mansingh and Smallman, 1966) and the solitary bee, *O. lignaria* (Bosch et al., 2010). In contrast, facultative diapause is initiated following the detection of environmental cues during a 'sensitive' stage, followed by diapause initiation later in the life cycle, or sometimes even in the subsequent generation. The majority of temperate insects enter a facultative diapause. Examples include the beetle *Microctonus vittatae* (Wylie, 1980), the wasp *Mormoniella vitripennis* (Schneiderman and Horwitz, 1958) and the cricket *Ephippiger ephippiger* (Hockham et al., 2001). Diapause can occur during any stage of ontogeny (from the embryo to adult). For any given species it is a life stage-specific response (Denlinger, 2002), although, a small proportion of species, such as the blow fly, *Protophormia terraenovae*, have the capacity to enter diapause in more than one life stage (Vinogradova, 1986).

The progression of diapause involves passing through a well conserved series of induction, initiation, maintenance and post-diapause quiescence (Tauber and Tauber, 1976; Denlinger, 2002; Koštál, 2006). This process is summarised in Figure 1.2.

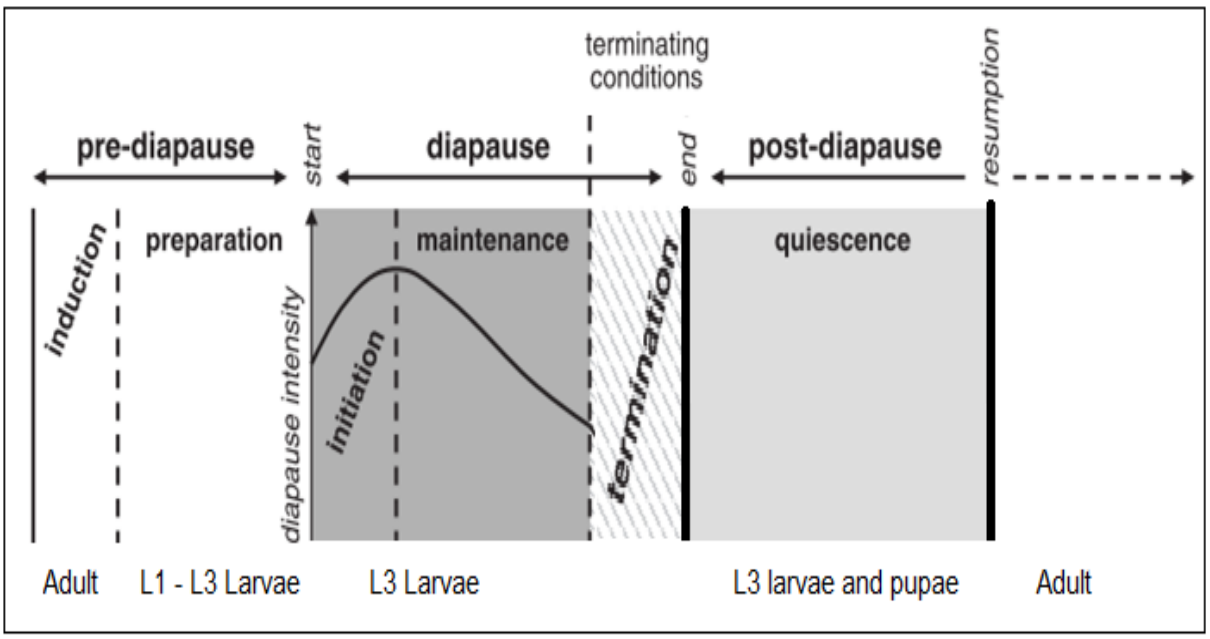

Figure 1.2 The progression of diapause through induction, preparation, initiation, maintenance and quiescence. The life stages associated with each phase are shown for the blow fly, *Calliphora vicina*. The curve represents the intensity of diapause associated phenotypes from initiation to maintenance. Adapted from Koštál (2006).

The conserved physiological states associated with diapause are possibly a result of the early appearance of diapause in insect evolution (Pullin, 1996), although recent metabolomics studies indicate that diapause is a result of convergent evolution and unlikely to be monophyletic (Ragland et al., 2010). Nevertheless, the stages illustrated in Figure 1.2 are experienced by the majority of diapausing insects (Tauber and Tauber, 1976; Denlinger, 2002; Koštál, 2006).

Hereafter, diapause will be discussed as a facultative response to adverse winter conditions. This is the most widely expressed form of diapause and the focus of this thesis. The phases of diapause are covered in more detail in the following section.

1.5 The phases of diapause

1.5.1 Induction

Diapause is induced when a sensitive stage in the life cycle detects a changing environmental cue, known as the token stimuli, indicative of advancing winter conditions (Lees, 1955). The sensitive stage can occur well in advance of the life stage that enters diapause (Tachibana and Numata, 2004a; Kostál, 2006; Salminen and Hoikkala, 2013). For example, in the aphids, *Myzus persicae* and *Megoiira viciae*, it is the grandparent generation that first experiences the token stimuli (Mousseau and Fox, 1998). In *C. vicina* it is the parental generation that detects the signal, while diapause is initiated by third-instar larvae of the subsequent generation (Saunders, 1987; Vaz Nunes and Saunders, 1989).

The token stimuli can either be a limiting resource availability, such as food or water scarcity, or a changing abiotic factor, such as low temperature (Tauber and Tauber, 1976). Within the temperate zone, the dominant cue is changing photoperiod (Mousseau and Dingle, 1991; Denlinger, 2002). Photoperiod has provided a robust indicator of changing seasons throughout evolutionary time (Saunders, 1997a, 2013). Typically, a photosensitive stage in the life cycle has the capacity to count day (or night) length, via a circadian clock mechanism (Tauber and Kyriacou, 2007; Kyriacou et al., 2008; Ikeno et al., 2010; Hahn and Denlinger, 2011; Saunders, 2013) often located in the brain (Numata et al., 1997). The specific photoperiod is termed the critical day length (CDL). The CDL is signified as inducing diapause in 50% or more of the population (Tauber et al., 1986). Once a threshold number of CDLs has been detected there is a neurohormonal output signifying the induction of diapause. For species entering adult diapause, the synthesis of juvenile hormone is often associated with switching from continued development to diapause (Denlinger, 2002; Ikeno et al., 2010). For larval and pupal life stages, there is often a shut-down of the brain-prothoracic gland axis which blocks production of ecdysteroid, a moulting and development

hormone bringing development to a halt and inducing diapause (Mousseau and Dingle, 1991; Denlinger, 2002; Hahn and Denlinger, 2011).

Following detection of the CDL, the induction of diapause is aborted in *C. vicina* if adults are exposed to temperatures exceeding 25°C (Vinogradova, 2011). Mousseau and Dingle (1991) suggest that high temperatures have the potential to interrupt the programming of diapause for the majority of species. It is believed that accelerated development under higher temperatures does not provide sufficient time to process diapause inducing cues (Mousseau and Dingle, 1991). For example, the adult mosquito, *Aedes atropalpus*, must experience nine diapause inducing CDLs for diapause to be induced (Beach, 1978). Under elevated temperatures adult development is completed in under nine days, and eggs of the next generation do not enter diapause (Beach, 1978). Adult temperature can also affect the proportion of individuals entering diapause, and the duration of diapause (Saunders, 1987; Tachibana and Numata, 2004b). This modifying effect of temperature could have important consequences as climate change continues, and temperature and photoperiod cues lose synchrony. Sensitive stages of the life cycle are increasingly likely to be exposed to higher late-autumn and early-winter temperatures (Bale and Hayward, 2010).

For many species there is a latitudinal variation in the specific CDL required to induce diapause. For example, *C. vicina* populations in southern England (51°N) have a CDL of 14.5 h, while in Northern Finland (65°N) it is 16 h (Vinogradova, 1986; McWatters and Saunders, 1996) (Figure 1.3).

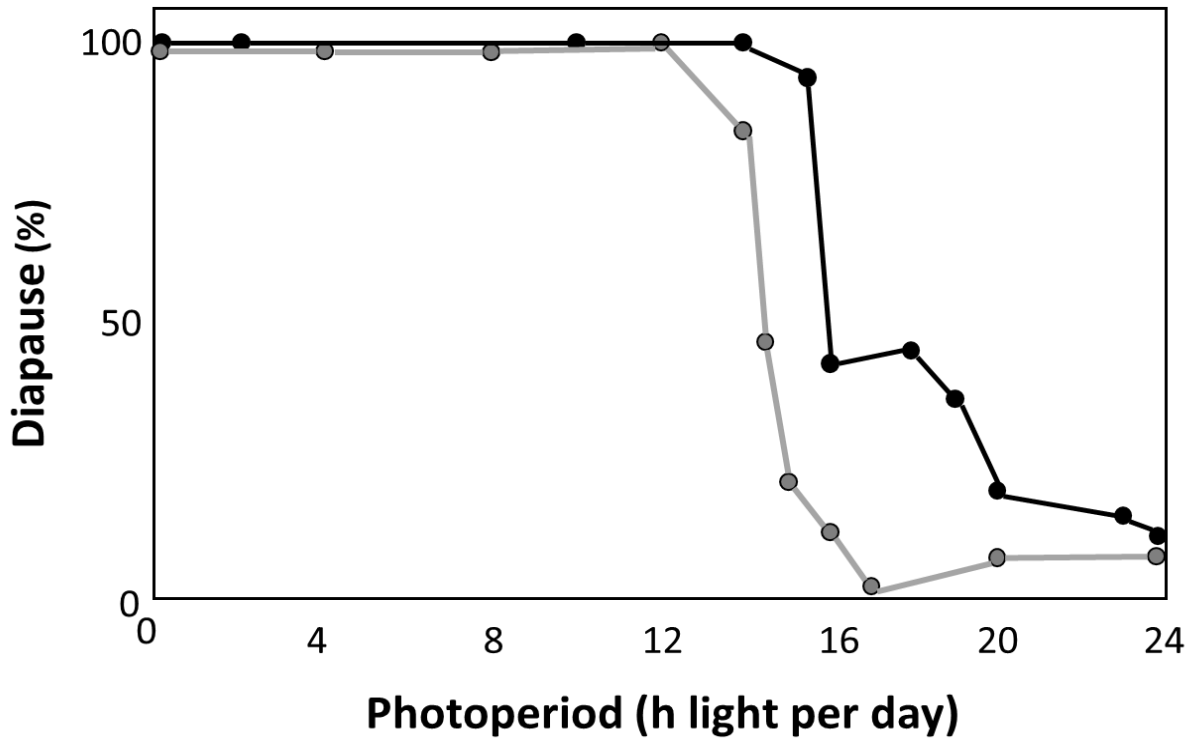

Figure 1.3 Photoperiodic response curves for two geographic strains of *C. vicina* showing the percentage of larvae that enter diapause. The strains are from 51°N (black line) and 65°N (grey lined). Adapted from McWatters and Saunders (1996).

1.5.2 Preparation

Providing the programming of diapause is not interrupted, induction is followed by diapause preparation. The preparation stage is signified by physiological and behavioural changes that assist in the initiation of diapause (Koštál, 2006). Behavioural changes can include locating a suitable overwintering site, group aggregation and increased feeding (Tauber et al., 1986; Danks, 2006; Wheeler and Cardé, 2013). Increased feeding facilitates the accumulation of the carbohydrate, fat and protein reserves laid down to survive the winter, and to aid in post-winter development (Saunders, 1997b; Denlinger, 2002; Xue et al., 2002). As a result of increased feeding, the body weight of some species can almost double during diapause preparation (Tzanakakis, 1959; Denlinger, 2002; Koštál, 2006), but this is not true of all insects (Saunders, 1997b).

Feeding rate often correlates with the proportion of individuals that enter diapause (Danks, 1987; Saunders, 1997b). Undernourished individuals will avert diapause and attempt to complete an additional generation. Increased feeding is typically followed by a period of starvation and gut evacuation to exclude possible sites of internal ice formation (Somme, 1999). Once preparation is complete diapause itself is initiated.

1.5.3 Initiation

Diapause initiation is a gradual process that commences when the diapause-specific life stage is reached. There is a measured decrease in metabolism and respiration, a specific pattern of gene and heat shock protein (HSP) expression (Figure 1.4), and a gradual increase in cold hardiness, as stress response mechanisms are implemented (such as molecular chaperones and cryoprotective compounds) (Denlinger, 2002; Hayward et al., 2005; Koštál, 2006; Hahn and Denlinger, 2011).

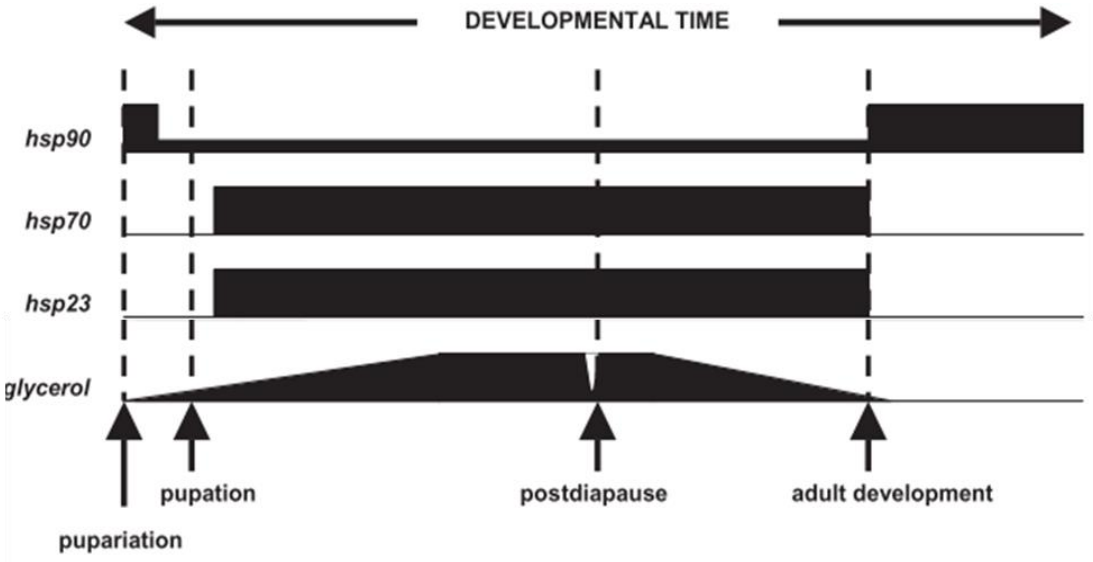

Figure 1.4 The temporal expression patterns of key diapause-associated genes and glycerol content from the time of pupariation through adult development in the flesh fly, *S. crassipalpis.* Adapted from Hayward et al. (2005).

The gradual changes associated with the progression of pupal diapause have been investigated in detail in the flesh fly, *S. crassipalpis* (Hayward et al., 2005). The early stages

of diapause are associated with a reduction in heat shock protein 90 (HSP90), and the up-regulation of HSP23 and HSP70. This is followed by a gradual increase in glycerol content. These changes are sustained throughout diapause and also during post-diapause quiescence.

Changes in respiration during diapause have been investigated in the flesh fly, *Sarcophaga argyrostoma*, in which diapausing pupae reduce oxygen consumption by 90% compared to their non-diapause counterparts (Figure 1.5) (Denlinger, 1972).

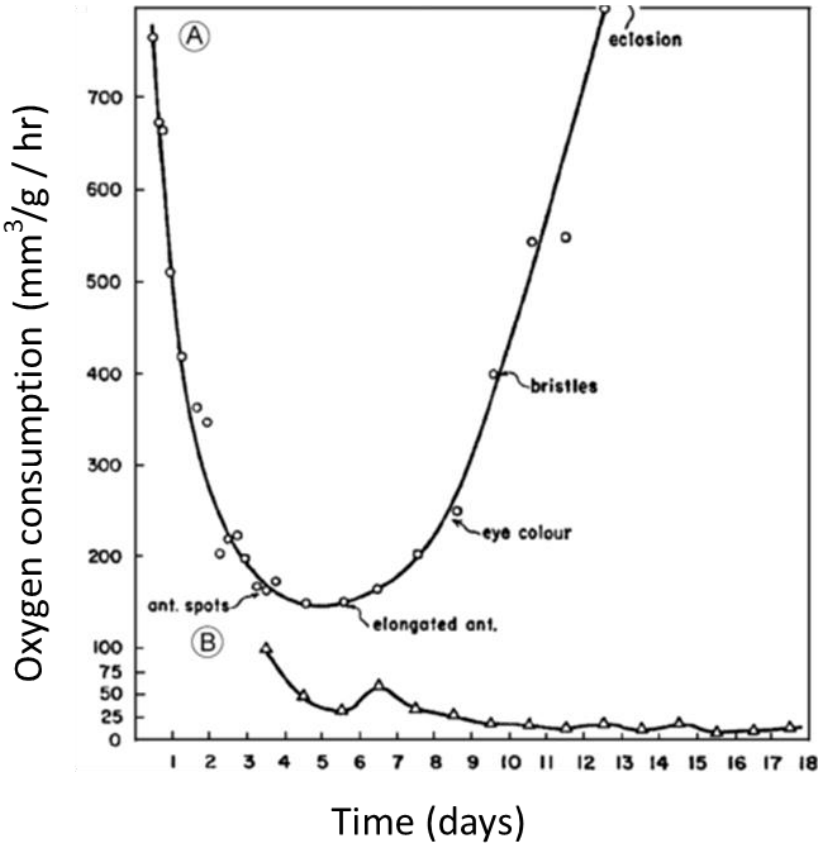

Figure 1.5 The oxygen consumption for non-diapause (A) and diapause (B) *Sarcophaga argyrostoma* from the time of pupariation until the time of adult eclosion. Different stages of development are identified for non-diapause pupae. Adapted from Denlinger (1972).

The low metabolic rate is achieved by locating a thermally stable, low temperature location. For species such as the Colorado potato beetle, *Leptinotarsa decemlineata* (de Kort, 1990),

there is also the shut-down of biochemical and physiological systems, such as flight and digestive tissue, which then have to be synthesised once diapause is completed.

Diapause initiation can also be signified by distinct morphological adjustments, such as colour changes as observed in the southern green stink bug, *N. viridula* (Musolin and Numata, 2004), but this is not always the case (Saunders et al., 1986). During the early stages of diapause there is often sensitivity to changing environmental conditions. For example, following the maternal induction of diapause, *Lucillia sericata* larvae must experience temperatures below 20°C, and a short-day photoperiod, for successful initiation of diapause (Tachibana and Numata, 2004a;, 2004b). Larval exposure to high temperatures and long-day photoperiod will result in diapause being aborted. A similar response is observed in numerous other species, including *C. vicina* in which larvae will abort diapause if exposed to temperatures above 15°C (Vaz Nunes and Saunders, 1989).

1.5.4 Maintenance

The maintenance stage represents diapause in its true sense. At this time the individual has entered an alternative form of development with its own unique physiology and energy production (Denlinger, 2002; Koštál, 2006; Michaud and Denlinger, 2007). Recent metabolomics studies have revealed that diapause is often associated with a decrease in Krebs cycle intermediaries, and the up-regulation of metabolites associated with glycolysis (such as glycerol, glucose, alanine and pyruvate), the process of anaerobic energy production (Michaud and Denlinger, 2007; Colinet et al., 2012; Zhang et al., 2012; Johnson, 2013). These changes signify that the Krebs cycle is brought to a halt to prevent the accumulation of the toxic end product, lactic acid, and instead favour glycolysis. This has non-toxic end products, and can continue under anaerobic conditions that may be experienced over the winter months (Johnson, 2013).

During maintenance, the individual is less responsive to external stimuli such as temperature and photoperiod. The metabolic rate is reduced and stress responsive mechanisms are fully

implemented (Denlinger, 2002). The maintenance stage can last anything from several weeks to several years, such as in the Yucca moth, *Prodoxus y-inversus*, in which diapause can reportedly last for up to 17 years (Powell, 1992).

1.5.5 Termination

Diapause can end in response to environmental stimuli such as chilling, freezing or photoperiod. Alternatively, development can be resumed spontaneously (Tauber et al., 1986). Species relying on an environmental stimulus to break from diapause often require species-specific conditions to be encountered. For example, the mosquito *Wyeomyia smithii* is sensitive to photoperiod (Emerson et al., 2010), and the parasitoid *Colpoclypeus florus* is sensitive to chilling (Milonas and Savopoulou-Soultani, 2000). The termination of diapause does not necessarily represent the point of resumed development. Instead, most species enter a period of post-diapause quiescence (Koštál, 2006).

1.5.6 Post-diapause quiescence

During post-diapause quiescence the cold tolerance mechanisms associated with diapause are still in place, diapause associated genes continue to be expressed and metabolism continues to be kept at a minimum (Figure 1.4) (Hayward et al., 2005). Post-diapause quiescence can be maintained for weeks to months, with development being resumed either spontaneously, or following detection of an environmental stimulus indicative of spring (Koštál, 2006). For *C. vicina*, development is resumed spontaneously if low temperatures are sustained, or if environmental temperatures exceed the developmental threshold of 15°C (Vaz Nunes and Saunders, 1989). Once post-diapause quiescence has completed the cold tolerance associated with diapause is lost (Hayward et al., 2005).

Post-diapause quiescence is thought to have evolved to allow synchronous emergence in spring across cohorts that may have had staggered entry into diapause, and terminated

diapause at slightly different times during winter. They are then primed for emergence under appropriate temperature cues once favourable conditions have returned (Koštál, 2006).

1.6 Diapause and cold hardiness

A close association between diapause and increased cold hardiness has been observed in many species, such as *Drosophila melanogaster* and *A. atropalpus* (Mansingh, 1974; Pullin et al., 1991; Manrique et al., 2012; Murata et al., 2013). Other examples include adults of the firebug, *Pyrrhocoris apterus* (Kodrík et al., 1995), pupae of the flesh fly, *S. crassipalpis*, (Lee and Denlinger, 1985), adults of the Coleoptera, *Aulacophora nigripennis* (Watanabe and Tanaka, 1998), and third-instar larvae of *C. vicina* (Johnson, 2013). Increased cold hardiness is achieved through the implementation of numerous stress response mechanisms that coincide with the initiation of diapause (these will be covered in more detail in the subsequent section) (Zachariassen and Husby, 1982; Koštál and Simek, 2000; Michaud and Denlinger, 2007; Rinehart et al., 2007). For a number of species the relationship between diapause and cold hardiness is less clear-cut. In the Coleoptera, *Dendroides canadensis*, a short-day photoperiod increases cold hardiness but does not induce diapause (Horwarth and Duman, 1983). For the European corn borer, *Ostrinia nubilalis*, a short-day photoperiod induces diapause but does not increase cold hardiness (Denlinger, 1991). There is no difference in the cold tolerance of diapause and non-diapause larvae of the green bottle, *L. sericata* (Ring, 1972). In the mosquito, *Aedes albopictus*, diapause is induced by detection of a specific CDL while cold hardiness is temperature-controlled (Hanson and Craig, 1994).

Hodkova and Hodek (2004) suggested that even in examples where diapause and cold hardiness are expressed simultaneously, it cannot be assumed that they are part of the same stress response mechanism. It is possible that they are induced over the same timeframe in response to independent environmental stimuli. Furthermore, even if they are part of the same stress response, it is possible that mechanisms providing stress tolerance, first evolved as a part of the diapause response, and only later proved beneficial in cold

adaptation. This was postulated by Pullin (1996) who suggested that diapause arose as a response to high temperatures in tropical locations, but proved advantageous in surviving the cold when insects dispersed to higher latitudes.

For the majority of species it is believed that diapause is a prerequisite for increased cold hardiness, and the two phenotypes are usually recognised as being part of the same overwintering response (Denlinger, 1991, 2002; Koštál, 2006; Denlinger and Lee, 2010).

1.7 Classes of cold hardiness

According to Salt (1961) all insects can be classified as either freeze tolerant or freeze avoiding. This is based on their ability to withstand extracellular ice formation. Upon gradual cooling, an insect will eventually reach a point of spontaneous freezing, referred to as the supercooling point (SCP) (Salt, 1961; Block, 1982). The SCP is identified as an exothermic heat output at the precise moment that water particles turn to ice, (Figure 1.6).

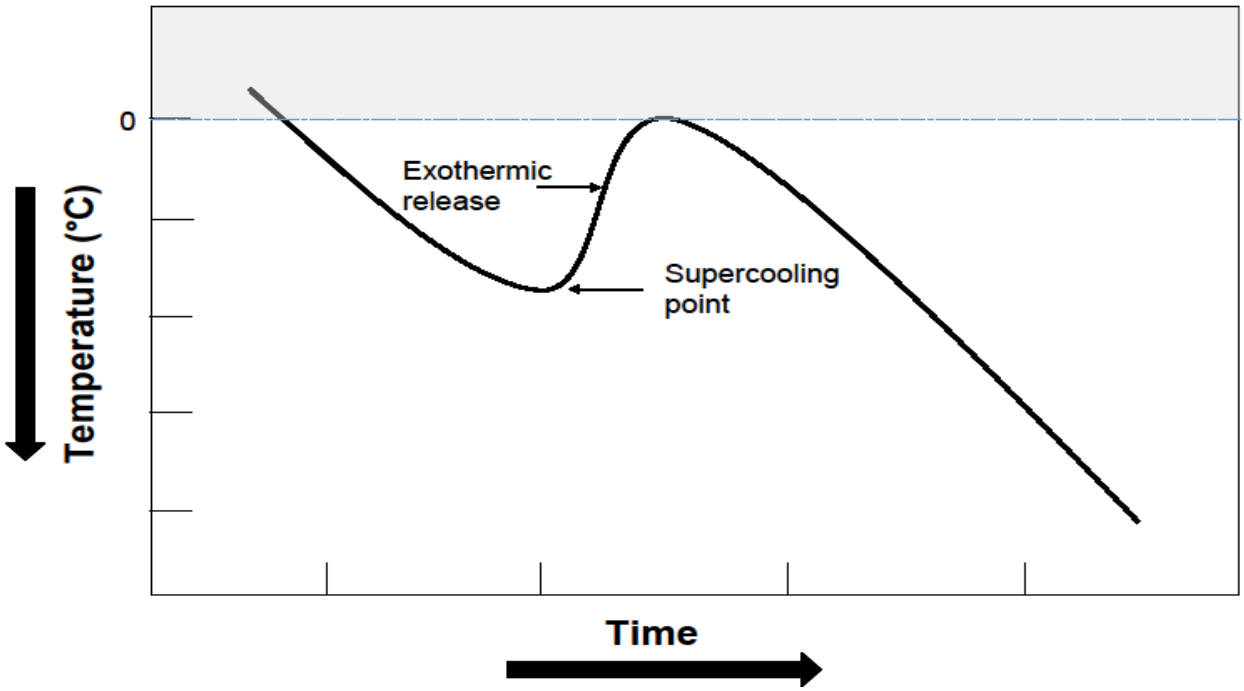

Figure 1.6 Expression of the supercooling point (SCP) as a hypothetical insect is gradually cooled. The supercooling point represents the moment of internal ice formation, which is detected as an exothermic event.

Freeze tolerant insects are capable of surviving prolonged exposure to temperatures below the SCP, while freeze avoiding insects experience complete mortality if temperatures reach the SCP (Bale, 1996, 2002).

1.7.1 Freeze tolerant insects

Freeze tolerant insects are typically found at high latitude locations that regularly experience extreme low temperatures (Bale, 1996; Sinclair, 1999). Their principle survival mechanism is to promote ice formation at relatively high sub-zero temperatures (usually above -10°C), through the accumulation of ice nucleating agents (INAs) (Zachariassen and Hammel, 1976; Ring, 1982; Zachariassen et al., 2004). The advantage of spending winter in a frozen state, is that providing temperatures remain below the SCP, internal temperatures remain stable. INAs were first isolated in hemolymph of the hornet, *Vespula maculate* (Duman and Patterson, 1978). Since this time INAs have been identified in numerous plant and animal groups (Storey and Storey, 1985, 1992; Ansart and Vernon, 2003; Márquez et al., 2006).

INAs are low molecular weight proteins that facilitate the slow and controlled formation of extracellular ice crystals (Figure 1.7) (Storey and Storey, 1992).

The formation of extracellular ice promotes the osmotic transport of water out of cells, reducing the potential of intracellular freezing damage (Zachariassen and Hammel, 1976; Storey and Storey, 1992; Bale, 1993).

Once freezing has occurred, the individual is capable of surviving exposure to extreme low temperatures. For example, *S. ribesii*, with an SCP of -6°C, exhibit 70% survival following -35°C exposure (Hart and Bale, 1998). Sinclair (1999) further divided the freeze tolerant group into four distinct strategies based on SCPs and the lower lethal temperatures of survival: partially freeze tolerant; moderately freeze tolerant; strongly freeze tolerant: and freeze tolerant with low SCPs.

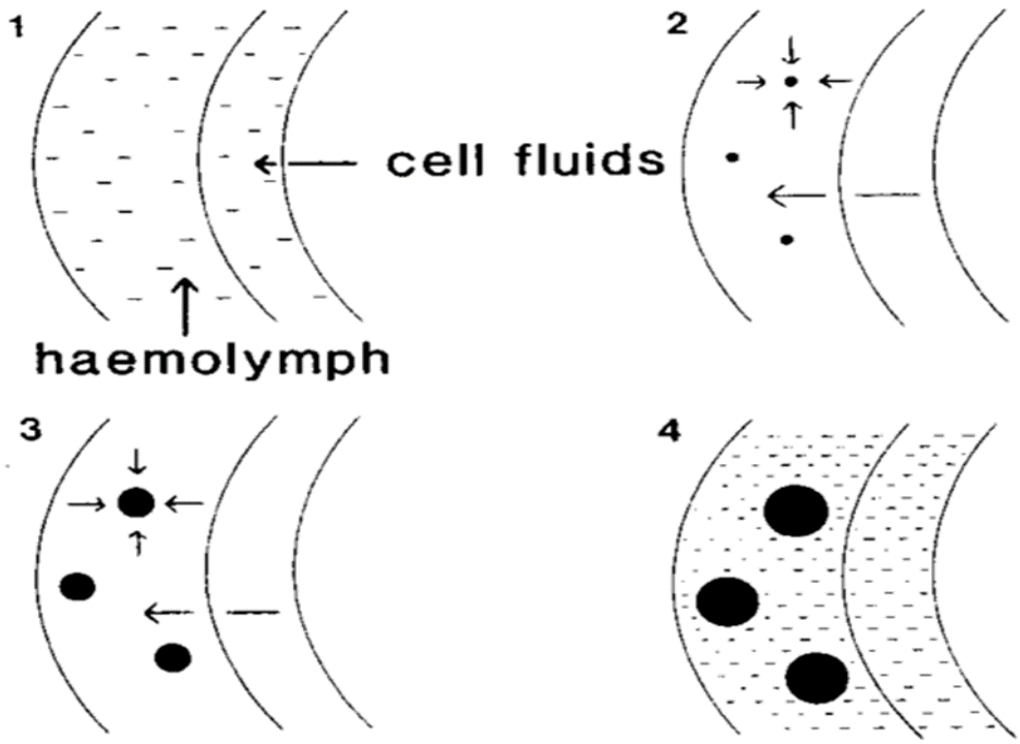

Figure 1.7 The role of ice nucleating agents (INAs) in promoting extracellular ice formation. Over the summer months (1) the hemolymph and cell fluids are in osmotic equilibrium. (2) INAs promote freezing in the hemolymph when sub-zero temperatures are experienced over the winter. (3) The size of the ice mass grows as temperatures continue to be lowered, while water moves out of the cell through osmosis. (4) The insect overwinters in a protective state where the ice crystals have formed in the hemolymph and intracellular spaces are protected from the direct effects of ice-crystal damage. Adapted from Bale (1996).

With the exception of INA production, the cold tolerance mechanisms exhibited by freeze tolerant and freeze avoiding insects are very similar. These mechanisms will now be covered in more detail in relation to freeze avoiding insects.

1.7.2 Freeze avoiding insects

The majority of temperate insects in the Northern Hemisphere are freeze avoiding, and survival is dependent on temperatures remaining above the SCP (Bale, 1996, 2002). To prevent freezing, potential INAs (including dust and food particles) are excluded during a period of gut evacuation (Salt, 1961). This is often followed by the synthesis of low molecular weight molecules, such as sugars or polyols, capable of lowering the internal freezing point

of water through a process of supercooling (Zachariassen, 1985; Carrasco et al., 2012). Supercooling is a result of these molecules remaining viscous at low temperatures, and inhibiting freezing, by competing with water molecules to form hydrogen bonds at sites of ice nucleation (Zachariassen et al., 2004). The limit of an individual's supercooling capacity is measured using the SCP (Figure 1.6).

The first supercooling compound to be identified was glycerol, in the silk moth, *H. cecropia* (Wyatt and Meyer, 1959). Glycerol is now recognised as an important cryoprotective compound for many overwintering insects. This is due to its ability to stabilise proteins, prevent intracellular mechanical damage and maintain the transmembrane flux of water at low temperatures (Ring and Tesar, 1981; Michaud and Denlinger, 2007; Hou et al., 2009; Denlinger and Lee, 2010). Cryoprotective compounds such as glycerol, sorbitol and mannitol are synthesised from glycogen before the onset of low temperatures, and then resynthesised once favourable conditions have returned, habitually coinciding with diapause initiation and termination (Denlinger and Lee, 2010).

Cryoprotectants are often considered to be either sugars or polyols, but, it is possible that amino acids are equally important in enhancing low temperature tolerance. Proline is the dominant metabolite in cold acclimated larvae of the fruit fly, *Drosophila melanogaster*; which is a freeze avoiding species that experiences complete mortality following direct exposure to -5°C (Koštál et al., 2011a). The larvae can however tolerate 50% of their body tissues being frozen at -5°C providing proline content is artificially increased (Koštál et al., 2012). Similarly, proline is the dominant metabolite in diapausing larvae of the drosophilid fly, *Chymomyza costata* (Kostál et al., 2011b). Diapause, but not non-diapause larvae, are able to survive submersion in liquid nitrogen (-196°C), making them one of the most cold hardy of all insects (Kostál et al., 2011b). Survival of non-diapause larvae following liquid nitrogen exposure increased from 0% to 36% when proline content was artificially increased (Kostál et al., 2011c). These studies confirm that proline has the potential to increase cold hardiness, but,

they do not uncover the role of proline at low temperatures, and it remains unknown whether proline is a common cryoprotectant used by other species.

The most frequently encountered amino acid in cold hardy life stages is alanine (Rivers et al., 2000; Li et al., 2001; Michaud and Denlinger, 2007; Overgaard et al., 2007; Johnson, 2013). *S. crassipalpis* exhibit a nine-fold increase in alanine when in diapause (Michaud and Denlinger, 2007), while there is a 2.5-fold increase in diapause larvae of *C. vicina* (Johnson, 2013). Alanine has also been associated with enhanced cold hardiness in previous studies on the gall fly, *Eurosta solidaginis* (Churchill and Storey, 1989) and *B. antarctica* (Michaud et al., 2008). Alanine accumulation in response to seasonal low temperatures in the fall webworm, *Hyphantria cunea*, was not associated with an increase in cold tolerance (Li et al., 2001). It is possible that alanine is up-regulated to assist in cold protection, or alternatively that it accumulates as a by-product of other physiological responses, such as the synthesis of protein and glucose, or as a by-product of glycolysis (Michaud and Denlinger, 2007; Johnson, 2013). At present the role of alanine, and other amino acids, in cold tolerance remains unclear.

Other low temperature adaptations include the synthesis of antifreeze proteins (AFPs). AFPs are small entities that inhibit ice formation through attachment to the outside of potential INAs (Zheng et al., 2011). While AFPs are usually found in freeze avoiding insects, they are present in a number of freeze tolerant insects such as *D. canadensis* (Duman et al., 1998). Another adaptation utilised by both freeze avoiding and freeze tolerant insects is the production of HSPs. These act as molecular chaperones by binding to other proteins (Rinehart et al., 2007). Additional adaptations include production of aquaporins and proteins that maintain water transportation between cells at low temperatures (Philip et al., 2008). Many insects also exhibit modifications to the lipid membrane bilayer (Colinet et al., 2012). An overview of how the different cold hardiness mechanisms are utilised by freeze avoiding and freeze tolerant insects is presented in Figure 1.8.

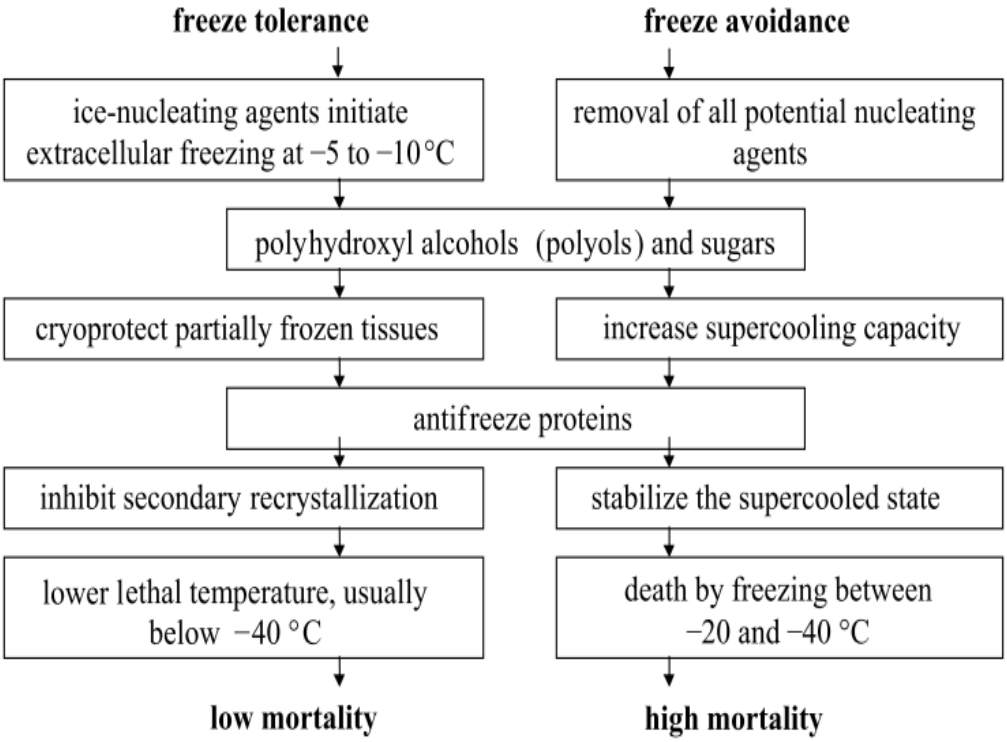

Figure 1.8 The low temperature mechanisms utilised by freeze tolerant and freeze avoiding insects. Adapted from Bale (1996, 2002).

At temperatures below the SCP freeze avoiding insects will experience freeze-induced damage, as a direct consequence of ice formation damaging membranes and proteins. An important consideration for freeze avoiding insects is the cause of mortality temperatures above the SCP (Bale, 1996).

1.7.3 Mortality at low temperatures

Cold induced damage can be classified as either direct chilling injury or indirect chilling injury. Direct chilling injury occurs over relatively short time scales (i.e. minutes) in response to acute cold exposure to temperatures above the SCP (Denlinger and Lee, 2010). It is a response to macromolecules and enzymes losing function at low temperatures, and a loss of membrane fluidity (Denlinger and Lee, 2010). Indirect chilling injury occurs over longer time scales (i.e. days to weeks) following chronic cold exposure to temperatures well above the SCP.

The recognition that freeze avoiding insects succumb to mortality at temperatures above the SCP led Bale (1996) to further divide the freeze avoiding classification into four different groups based on the varying ability of species to tolerate exposure to low temperatures (Table 1.1).

Table 1.1 Classification system of freeze avoiding insects proposed by Bale (1996, 2002).

Classification	Characteristics	Example
Freeze avoiding	Killed by ice formation but display little or no mortality at temperatures above the SCP	Autumnal moth, *Epirrita autumna*
Chill tolerant	Display a high degree of low temperature tolerance but succumb to mortality at temperatures above the SCP (circa -20°C)	Beech weevil, *Rhynchaenus fagi*
Chill susceptible	Display a relatively wide thermal range but succumb to mortality following exposure times of minutes to hours at temperatures well above the SCP	Green peach aphid, *M. persicae*
Opportunistic survival	Possess very limited ability to tolerate temperatures outside of the optimal thermal range and experience mortality following brief (minute) exposures to low temperatures	The house fly, *Musca domestica*

An ecologically relevant measure of cold hardiness is the Lethal Time (LT). LT is an index that allows assessment of cold induced mortality at temperatures above the SCP. Removing individuals at regular intervals from a sub-zero temperature naturally experienced *in-situ* can be used to determine the time taken to induce 10% (LT_{10}), 50% (LT_{50}) and 90% (LT_{90}) mortality. This is a popular technique that allows comparison between treatments and species (Hughes et al., 2009; Bürgi and Mills, 2010; Koštál et al., 2011b; Morey et al., 2012). While the SCP is not an ecologically relevant measure of cold hardiness (Bale, 1987), it does provide a useful comparative index and a positive correlation often exists between cold hardiness and supercooling capacity (Renault et al., 2002).

1.8 Activity at low temperatures

An ecologically relevant measure of temperature tolerance for active life stages is the identification of activity thresholds, beyond which individuals are unable to relocate to more favourable environments, locate food, reproduce or avoid predation (Hughes et al., 2010; Macmillan and Sinclair, 2011; Coombs and Bale, 2013). These thresholds are also of importance when predicting insect distributions and range shifts in response to climate change (Hazell et al., 2008).

As insects are cooled they pass through a conserved set of physiological responses. At first, activity becomes impeded and walking speed decreases until the ability to maintain coordinated movement is lost; this point is called the critical thermal minima (CT_{min}) (Cowles and Bogert, 1944). Beyond this point there is continued appendage movement until the individual reaches a point of chill coma, signified by a final appendage twitch (Mellanby, 1939). Beyond the point of chill coma the insect is in complete paralysis (Hazell and Bale, 2011). Chill coma is a reversible state and if temperatures increase movement and the ability to walk are regained. Should temperatures continue to decrease the individual will die. An insect passes through similar ecological thresholds at high temperature (Hazell et al., 2008). At first, coordinated movement is lost and this point is called the critical thermal maxima (CT_{max}). Beyond this point there is uncoordinated movement until the point of heat coma, signified by a final appendage twitch. Heat coma and CT_{max} are often closer together than chill coma and CT_{min}, and heat coma is usually synonymous with the upper limit of survival and is a non-reversible state.

1.9 Phenotypic plasticity

The thermal limits of survival are not static boundaries but plastic phenotypes influenced by environmental conditions experienced over preceding hours, days, weeks and years (Fischer and Karl, 2010). Upper and lower activity and survival thresholds are particularly susceptible

to the effects of thermal acclimation (Angilletta, 2009). Thermal acclimation includes, temperatures experienced over multiple life stages (ontogeny acclimation) or generations (cross generation acclimation), or over periods of days (gradual acclimation) or hours (rapid acclimation) (Angilletta, 2009; Colinet and Hoffmann, 2012; Hoffmann et al., 2013).

In general, there is a greater degree of plasticity in lower than upper thermal boundaries (Hoffmann et al., 2005; Rako and Hoffmann, 2006; Alford et al., 2012). Upper thermal thresholds range between 40 and 50°C, and plasticity in these limits rarely exceeds 1°C following acclimation (Chown and Nicolson, 2004; Alford et al., 2012; Hoffmann et al., 2013). It is believed that at temperatures above 50°C, intracellular oxygen becomes limiting and macromolecular structures become destabilised (Feder and Hofmann, 1999; Hoffmann et al., 2013). In contrast, there is considerable variation in lower survival thresholds of insects, ranging from around -16°C (CT_{min}) in the cold tolerant midge, *Diamesa meigen* (Kohshima, 1984) to above 20°C for tropical insects, such as the tsetse fly, *Glossina pallidipes* (Terblanche et al., 2007a). Low temperature thresholds often exhibit a high degree of plasticity. An example is the lower activity threshold of *Drosophila* spp. which on average is lowered by 1°C for every 4°C reduction in acclimation temperature (Gibert and Huey, 2011).

The magnitude and limits of phenotypic plasticity must be taken into consideration when predicting how organisms will respond to climate change. Phenotypic plasticity will only prove beneficial if an individual's evolutionary response is in line with the direction of environment change. For example, the beneficial acclimation hypothesis (Wilson and Franklin, 2002) suggests that the fitness of an individual is enhanced, if conditions are similar to those which they have previously experienced. Under this hypothesis, individuals may respond positively to climate change as they are progressively exposed to higher temperatures over multiple generations, with each generation gradually acclimating to the increasing temperature. However, Chown et al. (2010) suggest that phenotypic plasticity could also result in negative responses due to the unequal rate of warming across seasons. This could be of particular importance in the temperate zone, where late-autumn temperatures are predicted to increase

at a greater rate than winter temperatures (IPCC, 2013). The effect of this will be that life stages active over the autumn will experience gradually higher temperatures, as climate change continues, while winter generations will still have to endure periods of acute and chronic cold (Chown et al., 2010). It is already known, for species such as *C. vicina*, that adults under higher temperatures will produce fewer diapausing offspring (Bale and Hayward, 2010); however, it is not known what other phenotypes could be influenced by higher autumn temperatures.

1.9.1 Rapid cold hardening

An important acclimation response for a number of insect species is rapid cold hardening (RCH) (Lee et al., 1987). RCH is the ability to enhance survival of cold exposure through prior exposure, of minutes to hours, to a less severe temperature (Bale, 2002). Prior exposure of *S. crassipalpis* to 0°C for 2 h raised survival following short term exposure to -10°C from 5 to 99% (Lee et al., 1987). RCH is a reversible response that can occur during any stage of the life cycle and at any time of the year (Czajka and Lee, 1990). However it has not been confirmed whether diapause life stages, which are already expressing maximum cold tolerance mechanisms, have the capacity for RCH. It has been identified in 26 families belonging to nine insect orders (Denlinger and Lee, 2010; Owen et al., 2013) but it is noticeably absent from some species, including the marion flightless moth, *Pringleophaga marioni*, and the kelp fly, *Paractora dreuxi* (Sinclair and Chown, 2003; Terblanche et al., 2007b).

Original studies of RCH, in which organisms were held at a constant fixed temperature to enhance survival, were criticised as responses that would not occur in nature (Coulson and Bale, 1990). Nonetheless, the presence of a RCH under ecologically relevant cooling rates in both laboratory (Kelty and Lee, 2001; Powell and Bale, 2006; Everatt et al., 2012) and field (Koveos, 2001; Kelty, 2007) settings have established it as an ecologically relevant response. For some species that lack an overwinter diapause, such as *D. plexippus*, RCH is

believed to be an important overwintering mechanism (Larsen and Lee, 1994). Denlinger and Lee (2010) have highlighted a need for more studies to look at the ecological relevance of this response.

1.10 Climate change

Climate change is currently the single greatest threat facing all life on Earth (Thomas et al., 2004). Every ecosystem on the planet will be affected in some way (Walther et al., 2002; Parmesan and Yohe, 2003; Hickling et al., 2006; Bellard et al., 2012). How species respond will, in part, be determined by the proximity of environmental temperatures experienced to the thermal limits of survival, and the phenotypic plasticity of these thresholds (Chown et al., 2010; Franks and Hoffmann, 2012; Hoffmann et al., 2013). Accurately predicting species' responses requires an understanding of the environmental changes associated with climate change.

The fifth assessment report (AR5) of the Intergovernmental Panel on Climate Change (IPCC) concluded, with 95% certainty, that human actions have been the dominant cause of global climate change since around 1750, this being the start of the industrial revolution (IPCC, 2013). The period of geological time over which human actions have influenced the Earth's climate is referred to as the Anthropocene (Crutzen, 2002). One controversial hypothesis, proposed by Ruddiman and Thomson (2001), suggests that the Anthropocene could have started much earlier than previously suspected. According to this hypothesis, early humans started to influence the climate by increasing atmospheric CH_4 (methane) and CO_2 (carbon dioxide) concentrations as early as 6000 and 9000 years ago, respectively, through the introduction of agriculture (rice growing and animal husbandry), biomass burning and deforestation (Brook, 2009). While this hypothesis remains divisive, the role of anthropogenic greenhouse gas production in increasing global temperatures is unambiguous (IPCC, 2013).

Evidence that fossil fuel combustion increases the atmospheric concentration of $C0_2$ first emerged in the 1970s. This comes from atmospheric recordings in the South Pole and the Mauna Loa observatory, Hawaii (Figure 1.9) (Bacastow, 1976).

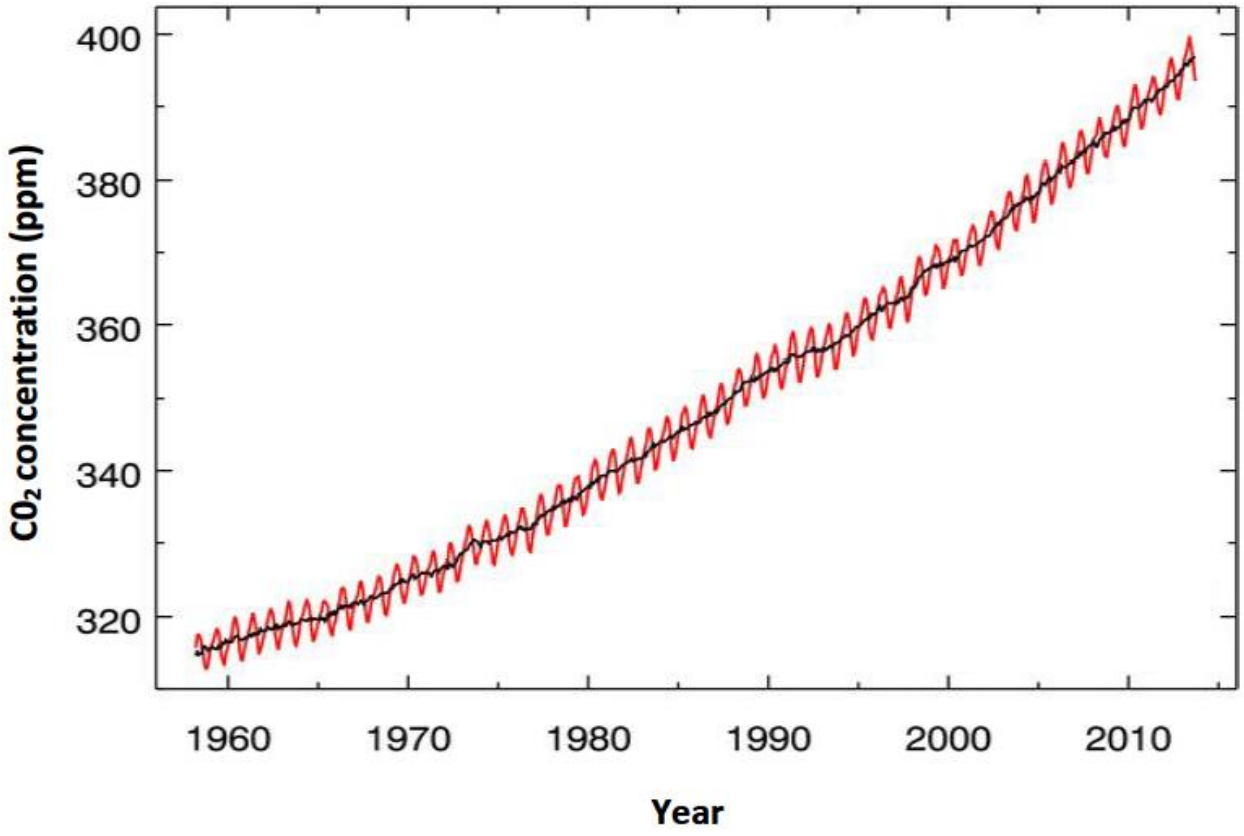

Figure 1.9 Monthly changes in CO_2 concentration (red line) between March 1958 and September 2013 at Mauna Loa Observatory, Hawaii. Black line represents seasonal averages. Adapted from NOAA (2013).

Recordings taken in September 2013 show that atmospheric CO_2 has increased to 393 ppm from a pre-industrial level of 278 ppm (NOAA, 2013). Over the same period, CH_4 and N_2O (nitrous oxide) have increased from 715 and 270 ppb to 1819 and 325 ppb, respectively (IPCC, 2013; NOAA, 2013).

The AR5 IPCC report predicts that atmospheric CO_2 will be in the range of 550 – 1,000 ppm by the end of this century (IPCC, 2013). The rise in atmospheric CO_2, and other greenhouse gases, has coincided with a global temperature increase of 0.76°C (since 1750) (IPCC, 2013). The speed of warming has accelerated in recent decades, with an average warming

of 0.12°C per decade over the last 50 years. Climate models project temperatures at the end of this century to be 1.5 - 4.0°C higher than the 1850 – 1900 average (Alley et al., 2007; Solomon et al., 2007). This rate of warming is unprecedented in at least the last 10,000 years.

An important facet of climate change is the unequal rate of warming across both space and time (Easterling et al., 2000). Warming has been greater in the Northern than Southern Hemisphere. Warming has also been greatest over autumn/winter months than summer months (Easterling et al., 2000; Solomon et al., 2007; IPCC, 2013). Also, night-time temperatures have increased at a greater rate than day-time temperatures. This unequal rate of warming creates a high degree of uncertainty when making future predictions for any given location. In Northern Europe, this is further complicated by a process of Polar amplification (Holland and Bitz, 2003). Polar amplification refers to the increased warming experienced in the Arctic due to positive feedback cycles, such as the ice albedo effect. According to the theory of polar amplification, accelerated warming could be experienced across Northern Europe and North America (Holland and Bitz, 2003). This is in response to Arctic temperature increasing by 5°C over the course of this century (Easterling et al., 2000; MacDonald, 2010). Within the temperate zone, temperatures across all seasons are increasing, yet, the winter period is still expected to experience periods of chronic cold. There is even the potential of more frequent high and low temperature fluctuations within Northern Europe in response to Arctic warming (Buckley and Kingsolver, 2012). The consequence for overwintering life stages is that while active life stages will encounter progressively higher temperatures in late-autumn and early-winter, overwintering life stages will still have to endure periods of prolonged winter cold.

1.11 *Calliphora vicina* (Robineau-Desvoidy)

The blow fly, *Calliphora vicina* is found in close association with human habitation throughout Europe, the Americas, Africa, Asia, Australasia and as far south as Kerguelen in the

southern Indian Ocean (Lebouvier et al., 2011). It is one of the earliest dipterids to emerge in spring in the UK and is recognised as both an important pollinator (Howlett, 2012) and decomposer of waste organic matter.

C. vicina has a rich and diverse history in biological studies (Vinogradova and Zinovjeva, 1972; Tu and Dickinson, 1996; Vinogradova, 2009; Chernysh and Gordja, 2012). Its ability to colonise corpses rapidly has established it as the primary insects in determining post-mortem intervals (the time from death to discovery of the corpse) in forensic entomology (Kaneshrajah and Turner, 2004). A combination of standard culturing techniques and an understanding of their life history makes *C. vicina* a popular study species in investigations of insect flight (Tu and Dickinson, 1996), circadian rhythms, photoperiodism (Saunders, 1997a; Nunes and Saunders, 1999), cold hardiness and diapause (Saunders and Hayward, 1998; Johnson, 2013). *C. vicina* has also been identified as a possible source of medicinally important drugs, such as antimicrobials (Chernysh and Gordja, 2012).

C. vicina populations at northern range boundaries are univoltine and exhibit an obligatory diapause (Vinogradova, 1986, 2009) while populations in the UK enter facultative diapause (Saunders, 1987). Parental adults induce diapause following detection of a specific CDL, while diapause is experienced by post-feeding third-instar larvae of the subsequent generation (Vaz Nunes and Saunders, 1989). The CDL for Birmingham populations (at 52.4°N, 1.9°W) is 14.5 hours. This day length (sunrise to sunset) is experienced around the 11th August, but, low level light sensitivity during twilight suggests diapause induction probably occurs slightly later in the year (Johnson, 2013). The life cycle of *C. vicina* is presented in Figure 1.10.

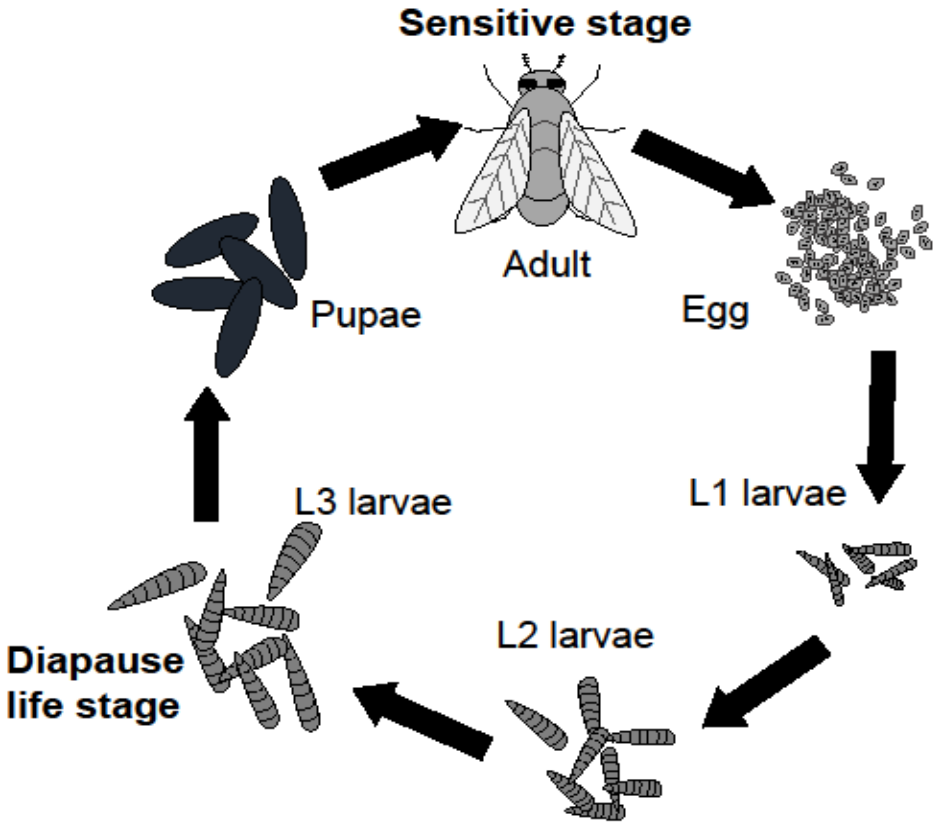

Figure 1.10 The life cycle of the blow fly, *Calliphora vicina*. Parental adults are the sensitive stage to the diapause inducing photoperiod while diapause is initiated by third-instar (L3) larvae of the subsequent generation.

While it is known that higher adult and larval temperatures can reduce the induction and duration of diapause, it is not known if these temperature thresholds are already being exceeded *in-situ*. Also, it is unclear if non-diapause life stages have sufficient cold tolerance mechanisms to survive the winter, following diapause aversion, or if higher temperatures associated with a reduced incidence are capable of reducing the cold hardiness phenotype of the diapause life stage. While *C. vicina* provides an ideal study system to investigate the potential impact of climate change, it also provides a useful model for unravelling responses to climate change in other temperate insects. Many characteristics of diapause in *C. vicina* are representative of a typical diapause programme experienced by temperate insects, including facultative photoperiodic induction by the parental generation, the modifying effect of temperature on the initiation of diapause, the increase in cold hardiness associated with

diapause and the expression of diapause during a non-feeding life stage (Tauber et al., 1986; Mousseau and Dingle, 1991; Denlinger, 2002; Bale and Hayward, 2010).

1.12 Summary and objectives

The main aims of this thesis are to (i) further our understanding of the regulation of diapause in blow fly, *Calliphora vicina*, under present and future climate conditions, and (ii), investigate the relationship between diapause and increased cold tolerance, and the role of amino acids in the expression of the cold hardiness phenotype associated with diapause.

It would be impossible and futile to catalogue systematically how individual species will respond to future climate change. Instead effort should focus on investigating general responses that are applicable to a wide range of species. For insect species, this has typically involved investigating responses to increased temperature, such as upper thermal thresholds being exceeded in tropical locations, and the alleviation of cold-induced stress in temperate locations (Crozier, 2003; Westgarthsmith et al., 2007; Terblanche et al., 2008; Jepsen et al., 2011; Musolin, 2012; Hoffmann et al., 2013). An area of insect biology that has received very little attention is the effect of climate change on overwintering life stages (Bale and Hayward, 2010; Sgolastra et al., 2011; Radchuk et al., 2013). For temperate insects, the overwintering life stage encounters periods of intense stress, prolonged and acute cold and limited nutrient availability. These conditions expose overwintering life stages to conditions at the very limits of survival, which are only endured through the implementation of highly specialised behavioural and physiological adaptions. Disruption to these adaptations, or a change in prevailing environmental conditions, could have unforetold impacts on the insect life cycle, ranging from a loss of synchrony with vital resources availability, to reduced fitness and increased mortality.

Higher temperatures are already known to reduce the duration and incidence of diapause for a number of species, including *C. vicina*. For *C. vicina*, it is the parental generation that will

encounter higher temperatures in autumn, as climate change continues, while diapause life stages will still have to survive periods of prolonged winter cold. At present, it is not known what effect adult acclimatisation to higher autumn temperatures has on the cold tolerance ability of the diapause life stage. In Chapter 2, the cross generation effect of parental temperature on the cold tolerance of diapause and non-diapause progeny is investigated. A cross generation loss of cold tolerance has not previously been identified for any insect species, and it would represent a novel and critical response to climate change.

The reduced diapause incidence and duration associated with higher temperatures also means that non-diapause life stages will increasingly encounter periods of low temperature as climate change continues. In chapter 3, the ability of non-diapause and diapause life stages to heighten cold tolerance through the process of RCH is investigated. RCH is recognised as a well conserved response, although, very few studies have investigated its ecological relevance. RCH provides an important adaptation in extending lower activity thresholds. For adult life stages, it is equally important that temperatures remain above thresholds at which locomotion can be maintained. In Chapter 4, the ecological limits of survival (CT_{max}, CT_{min}, heat coma and chill coma) are investigated, as is the effect of acclimation and photoperiod on extending these limits. Chapter 5 then uses four years of *in-situ* phenological data to identify the timing of diapause induction and termination in the Birmingham population of *C. vicina*. Yearly anomalies are then used to determine what environmental factors are most important in disrupting the timing of diapause.

The relationship between cold hardiness and diapause is investigated in Chapter 6, through establishing non-diapause and diapause selection lines under diapause inducing conditions. The cold hardiness of high and low diapause incidence lines are compared, to disentangle the role played by environmental conditions experienced during diapause induction (adults for *C. vicina*), from diapause initiation and the associated increase in cold hardiness. Finally, Chapter 7 uses a combination of dietary manipulation and metabolomics techniques to explore the role of amino acids in heightening cold tolerance during diapause.

The general discussion will then contextualise the findings from this thesis within the wider literature, focusing particularly on winter activity of terrestrial insects under future climatic conditions.

CHAPTER 2

CROSS GENERATION PLASTICITY IN COLD HARDINESS IS ASSOCIATED WITH DIAPAUSE, BUT NOT THE NON-DIAPAUSE, DEVELOPMENTAL PATHWAY

2.1 Abstract

The majority of temperate insects overwinter in a state of diapause, a pre-emptive response to winter conditions associated with increased cold hardiness. Diapause is often induced following maternal adult detection of an environmental cue signifying the onset of winter, while diapause is initiated in a subsequent life-stage/generation. Continued global warming will expose adults to higher late-autumn temperatures, while diapause life-stages will still experience prolonged winter-cold. The cross generation effect of temperature was investigated by acclimating adult *Calliphora vicina* to present day (15°C) and future (20°C) late-autumn conditions and assessing cold-hardiness in diapause (D15 and D20) and non-diapause (ND15 and ND 20) progeny.

A cross generation plasticity in cold hardiness was associated with diapause but not the non-diapause pathway. D15 larvae exhibited an enhanced ability to suppress internal freezing (SCP = -18.9 ± 0.9°C) compared to D20 (-15.3 ± 0.8°C), and displayed a greater tolerance of prolonged exposure to -4°C (LT_{50} 26.0 ± 1.0 days and 11.4 ± 1.1 days, respectively) and -8°C (5.1 ± 1.1 days and 3.0 ± 1.1 days, respectively). These changes were associated with a reduced glucose content in D15 (2.4 ± 0.3 g mg^{-1}) compared to D20 (3.0 ± 0.3 g mg^{-1}) larvae.

In conclusion, *C. vicina* adults exposed to warmer autumn conditions during diapause induction will produce larvae with a reduced cold hardiness capacity, which could negatively impact winter survival. Given that maternal regulation of diapause is common among temperate insects this could be a widespread phenomenon.

2.2 Introduction

Insects inhabiting the temperate zone experience yearly winter cycles of low temperatures, potentially freezing conditions and limited nutrient availability. To enhance the chances of survival during this period, insects either migrate to more favourable locations, which offer

sufficient resources for growth and reproduction, or remain *in-situ* where physiological and biochemical adaptations are implemented to assist winter survival (Tauber and Tauber, 1976; Danks, 1987, 2002). The majority of temperate insects utilise the latter strategy and enter a dormant state of diapause (Denlinger and Lee, 2010). The dominant induction cue for diapause is day length (photoperiod), which has provided a robust indicator of approaching winter conditions throughout evolutionary time (Saunders, 1997a, 2013). Induction occurs during a sensitive stage in the life cycle, and the CDL denotes a photoperiod which induces 50% diapause within a population (Tauber et al., 1986). The sensitive stage is species specific, and very often at an earlier life stage (or generation) to the one that enters diapause (Tachibana and Numata, 2004; Kostál, 2006; Salminen and Hoikkala, 2013). For example, in the blow fly, *Calliphora vicina* (Robineau-Desvoidy, 1830), the adult female (sensitive) stage experiences the CDL to induce diapause, while diapause initiation does not occur until the third larval instar of the subsequent generation (Saunders, 1987). This maternal regulation of diapause is commonly expressed in temperate insects. Thus, diapause is usually induced well in advance of winter and any exposure to cold stress (Tauber et al., 1986). The CDL typically increases with latitude to account for the earlier onset of winter conditions (Tauber and Tauber, 1972). In the UK, CDLs for *C. vicina* range between 14.5 to 15.5 hours (Saunders and Hayward, 1998), which places the timing of diapause induction around early September.

The use of pre-emptive environmental cues to programme diapause provides sufficient time to prepare for the arrival of adverse conditions (Hahn and Denlinger, 2011). This includes the accumulation of energy reserves, reduced metabolic activity, and locating to an overwintering site (Denlinger, 2002; Koštál, 2006). Once diapause is initiated there is often an increase in cold hardiness (Goto et al., 2001; Khodayari et al., 2013), associated with the implementation of stress response mechanisms including synthesis of blood sugars, polyols and amino acids (Koštál and Simek, 2000; Koštál et al., 2011a,b), anti-freeze proteins, HSPs (Rinehart et al., 2007), and alterations to the lipid membrane bilayer (Michaud and Denlinger, 2006). These

changes often coincide with an ability to lower the internal freezing point to a temperature known as the supercooling point (SCP) (Zachariassen and Husby, 1982; Zachariassen, 1985; Hahn and Denlinger, 2011), achieved through reduced water content (Zachariassen et al., 2004), removal of ice nucleating agents (Duman, 1982) and synthesis of blood sugars and ions (Bale, 2002). While it is recognised that the SCP is not an ecologically relevant measure of cold hardiness for the majority of insects (Bale, 1987), it does provide an indicator of physiological changes induced by different thermal conditions.

Very little is known about how species will respond to potential diapause disruption through climate change (Bale and Hayward, 2010). Generally, it has been hypothesised that insects will respond positively as winter cold is alleviated and growing seasons become longer (Bale et al., 2002; Walther et al., 2002; Musolin, 2012). Evidence for this includes the poleward range expansions of the southern green stink bug, *Nezara viridula* (Tougou et al., 2009; Musolin et al., 2010; Musolin, 2012), and the Lepidopteran, *Atalopedes campestris*, (Crozier, 2003), as well as the earlier spring emergence of many bee and butterfly species (Gordo and Sanz, 2005; Bartomeus et al., 2011; Altermatt, 2012). However, negative responses are equally well-documented, including heightened disease outbreaks in the Lepidopteran, *Boloria eunomia*, and rapid depletion of metabolic reserves in the bee, *Osmia lignaria* (Sgolastra et al., 2011; Radchuk et al., 2013). Climate change also threatens to decouple photoperiod and temperature cues (Bale and Hayward, 2010). For example, diapause is aborted following detection of the CDL in *C. vicina* if temperatures exceed 20°C for adults, or 15°C for larvae (Vaz Nunes and Saunders, 1989; Mcwatters and Saunders, 1998). Warmer autumn conditions at temperate latitudes (Williams et al., 2012), make this a real risk. Insects aborting diapause must then complete an additional generation to permit diapause induction later in the year, or risk a non-diapause life stage being exposed to winter cold (Bale and Hayward, 2010). To assume climate change will reduce cold-induced winter mortality is over-simplistic, as winters at temperate latitudes will clearly remain cold – indeed there is increasing evidence of more frequent extreme climatic events (Buckley and Kingsolver,

2012). Thus, understanding the immediate and future impact of climate change on diapause and winter survival is critical to forecasting changes in insect distribution and abundance.

A previously unrecognised threat is that while autumn temperatures may not be sufficiently high to abort diapause they may still influence the transfer of biological information (genetic and epigenetic) from parents to progeny. Specifically, elevated temperatures experienced by a parental population in autumn could potentially reduce the cold hardiness of their overwintering larvae. This response would only occur if cross generational plasticity exists in the cold hardiness phenotype (i.e. if adult thermal history influences larval cold tolerance phenotypes). It is already known that higher adult temperatures reduce diapause incidence and duration (Saunders and Hayward, 1998), and there is strong evidence in a range of organisms that the physiological history of the parental generation can influence the stress tolerance phenotypes of their progeny (Marshall, 2008; Castro et al., 2013; Plautz et al., 2013; Suter and Widmer, 2013). Such a cross generation effect would be of significant ecological relevance and further highlight the importance of including autumn temperature conditions in models of insect overwintering under climate change scenarios.

Against this background, this chapter investigated the effect of adult acclimation history on the cold hardiness of both diapause and non-diapause progeny of *C. vicina*. At the same time this chapter investigates the relationship between diapause and cold hardiness, by identifying how adult acclimation history affects: (1) the supercooling capacity, (2) cold tolerance, and (3) rapid cold hardening (RCH) ability (see section 1.9.1 for an explanation of RCH).

2.3 Materials and methods

2.3.1 The study species

C. vicina for experimental use in this chapter (and all subsequent chapters) were originally sourced from the University of Birmingham campus, UK (52.4°N, 1.9°W) in 2009 using olfactory-traps as presented in Figure 2.1.

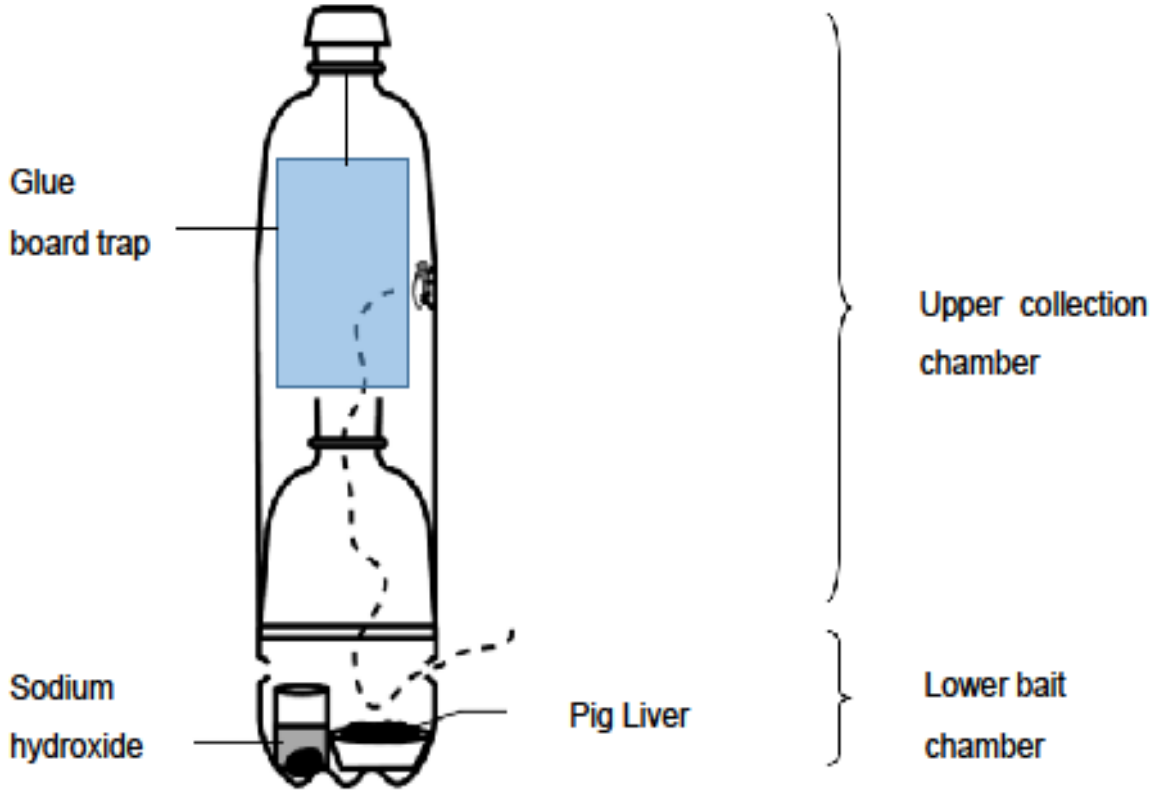

Figure 2.1 Olfactory fly trap with a lower bait chamber containing sodium hydroxide and pig liver to attract adult flies. Adults attracted to the bait chamber become trapped in the upper collection chamber. Designed by Hwang and Turner (2005) and adapted from Johnson (2013).

Traps were left in place at a deciduous forest-site (52.4°N, 1.9°W) for one week. Adult flies enter the bait chamber, feed and oviposit (lay eggs) on the liver, and then naturally fly through the inverted bottle lid and become trapped in the upper collection chamber. Egg laying (or oviposition) was subsequently removed, transferred on to an abundance of liver and gently wrapped in aluminium foil. The liver-parcel was then placed over 5 cm of finely

sieved sawdust in a breathable plastic container and held under complete darkness at 11°C to allow larvae to develop through to the adult life stage. At the time of eclosion (emergence) adults were removed and identified to species level. Individuals identified as *C. vicina* were retained for establishment of stock cultures. Typically, over 90% of emerged adults were *C. vicina*.

Stock cultures were regularly replenished with wild caught individuals and were maintained as outlined by Hayward and Saunders (1998). Individual stock cultures were established using approximately 300 newly eclosed mixed sex adults in 30 cm^3 cages. Cultures were held in light-tight shelving units under a long day-length photoperiod (LD 18:6 h) at 20°C. Sugar and water were provided *ad libitum* and 15 g of pig liver was provided as a protein source and site for oviposition on days (d) 4, 6, 8 and every day thereafter. This feeding method has been shown to synchronise ovarian development and to optimise production of the egg mass (Saunders, 1987). From d 12 onwards eggs were removed and held in an air-tight plastic container lined with damp absorbent paper (thereby creating <100% relative humidity) under constant darkness at 11°C for 48 h. High humidity induces a change in egg shape which increases strain on the chorion (outer) layer, making it easier for larvae to rupture their shells and emerge as first-instar larvae (Davies, 1950).

First-instar larvae were then transferred to an excess of liver, wrapped within aluminium foil over 5 cm of finely sieved sawdust, and held in breathable-plastic containers under constant darkness at 11°C. Feeding ceased after approximately 12 d, at which time third-instar larvae wandered into the underlying sawdust. Within 48 h of wandering, larvae were transferred to fresh sawdust and returned to 11°C in complete darkness until the time of adult eclosion. Newly eclosed adults were used for the establishment of both subsequent stock and experimental cultures.

2.3.2 Experimental cultures

Experimental cultures of adult flies were established in 30 cm^3 cages using approximately 300 mixed sex *C. vicina* on the day of mass eclosion with an abundance of sugar and water. Experimental cultures were maintained under controlled incubator conditions as follows:

- Long-day photoperiod (LD 18:6 h) at 15°C or 20°C to produce non-diapause larvae (abbreviated to ND15 and ND20, respectively).
- Short-day photoperiod (LD 12:12 h) at 15°C or 20°C to produce diapause larvae (abbreviated to D15 and D20, respectively).

Diapause larvae were also produced from cross temperature cultures established under short-day photoperiods by separating males and females at the time of eclosion and culturing them at either 15°C or 20°C. Males and females were combined after 10 d to create the four crosses presented in Table 2.1, all under short-day photoperiods.

Table 2.1 Cross temperature adult cultures for production of diapause *C. vicina* larvae. All adults were separated according to sex at eclosion, cultured under a short day photoperiod (LD 12:12 h) and crossed after 10 d.

Treatment	Male temperature (°C)	Female temperature (°C)	Temperature following Cross (°C)
♂20 × ♀20	20	20	20
♂20 × ♀15	20	15	15
♂15 × ♀20	15	20	20
♂15 × ♀15	15	15	15

For all cultures, liver was provided as a protein source and site of egg oviposition on day (d) four, six, eight and every day thereafter. From d 12 onwards eggs were removed from parental conditions within 18 h of oviposition and transferred to complete darkness under high humidity at 11°C. Removal of eggs within 18 h ensured a synchronous cohort of larvae which all experienced identical conditions. Thus, any subsequent variation in cold hardiness was a response to adult acclimation temperature.

Diapause and non-diapause larvae are morphologically indistinguishable, but diapause larvae can be identified as larvae not pupariating by d 30 post-oviposition (Saunders, 1987). The larval ring gland loses sensitivity to the brain neuropeptide, prothoracicotropic hormone, preventing larvae from developing to the pupal life stage (Richard and Saunders, 1987). For this reason non-diapause larvae were collected d 15 post-oviposition, while diapause larvae were identified by a delayed pupariation (Saunders, 1987) and collected d 30 post-oviposition. Unless stated otherwise, non-diapause and diapause larvae were used for experimental use on d 15 and d 30, respectively.

2.3.3 Supercooling capacity

Supercooling capacity was determined by attaching individual larvae to type K exposed wire thermocouples (Pico Technology, Cambridgeshire, UK) using a small amount of OecoTak adhesive glue (Oecos Ltd, Kimpton, Hertfordshire, UK). Individual larvae were then placed into 1 ml Eppendorf tubes (Sigma-Aldrich, Gillingham, Dorset, UK), which were placed in boiling tubes submerged in a programmable alcohol bath (two tubes per boiling tube) (Bale et al., 1984). The temperature was reduced by 0.5°C min^{-1} from 11°C to -30°C and the SCP detected by an exothermic output upon freezing (Figure 1.6). This was performed on larvae from all adult treatments. For diapause samples, SCPs were also recorded at the egg, L1 (1st instar larvae), L3-early (3rd instar), and pupal stages (d 0, 1, 12 and 50 post-oviposition, respectively), as well as for newly eclosed adults (i.e. on day of eclosion). (*n* = minimum of 26 per treatment).

2.3.4 Lethal time (LT)

The LT was determined for ND15, ND20, D15 and D20 larvae. Three groups of 10 third-instar larvae were placed in 10 ml glass vials containing 1 cm of sawdust, held in a programmable incubator at either -4°C or -8°C, and removed at 3 d intervals for the first 18 d and then 3 d or 6 d intervals until 50 d. Preliminary experimentation found 50 d to ensure complete mortality. Vials were removed and held at 11°C in darkness for 24 h then at 20°C

until eclosion. Larvae failing to eclose after 21 d were considered dead. Controls were maintained at 11°C for the duration of each experiment. A probit analysis was then used to estimate the time taken to kill 10% (LT_{10}) and 50% (LT_{50}) of the population. Methods used to investigate the LT are based on previous approaches (Saunders and Hayward, 1998; Hart et al., 2002; Hughes et al., 2009, 2010). (*n* = approximately 120 per treatment).

2.3.5 Rapid cold hardening (RCH)

The temperature giving rise to 80% mortality following a 2 h exposure period (the discriminating treatment - DT) (Lee et al., 1987) was determined following the plunge method (Nunamaker, 1993; Kelty and Lee, 2001). Individuals were placed in 50 ml test tubes and plunged into an alcohol bath (Grant LTD D6C, Grant Instruments, UK) at 1°C increments from -4° to -18°C for 2 h. Survival was assessed as successful development to the adult life stage for larvae held at 11°C.

RCH ability was then determined by:

- Holding larvae for 2 h at 0°C before transfer to the DT.
- Cooling larvae from 11°C to the DT at a rate of 0.5°C min^{-1}.

This was determined for ND15, ND20, D15 and D20 larvae (*n* = 60 per treatment). Controls were held in 50 ml test tubes at 11°C for the duration of each experiment.

2.3.6 Larval mass, and water and glucose content

Following measurement of whole-body wet mass to the nearest mg, ND15, ND20, D15 and D20 larvae were dried to constant mass for 2 d at 70°C. Dry mass was then recorded and water content calculated gravimetrically as a percentage of dry mass. Whole-body glucose content was determined using a Glucose (HK) Assay Kit (Sigma GAHK-20, Sigma-Aldrich, Gillingham, Dorset, UK). Samples of five L3 larvae from the four adult treatments were homogenized and diluted with deionised water. Glucose content was determined

spectrophotometrically by measuring absorbance of light at 340 nm (*n* = 80 for weight and water content; *n* = 20 for glucose content).

2.3.7 Statistical analysis

Analysis of SCPs, RCH, body mass, water content and glucose content data was undertaken using SPSS (v. 20.0, IBM, New York, USA). Data were first subjected to a Kolmogorov-Smirnov test to identify the distribution that best-described results. Data were then analysed using separate general linear models (GLMs) and significant differences between groups identified using the Bonferroni post-hoc tests with the alpha threshold set at 0.05. LT data were analysed using the statistical package Minitab 15 (Minitab, Coventry, UK). A probit analysis, based on Finney (1971), was used to determine the LT_{10} and LT_{50} by identification of non-overlapping upper and lower percentiles (± 95% fiducial limits) as in Hart et al. (2002).

2.4 Results

2.4.1 Supercooling capacity

The SCP data was normally distributed in larvae from 15°C and 20°C adults (F_{468} = 0.15; *P*=0.07).Acclimating adults to either 15°C or 20°C had no influence on the SCP of non-diapause larvae ($F_{1,52}$ = 0.03; *P*=0.87) (Figure 2.2).

The effect of adult acclimation on the supercooling capacity of non-diapause progeny was not investigated any further. There was an effect of adult acclimation on the SCP of diapause larvae, with D15 larvae (SCP = -18.9 ± 0.9°C) exhibiting significantly lower SCPs than D20 larvae (SCP = -15.3 ± 0.8°C) ($F_{1,54}$ = 9.5; *P*<0.01) (Figure 2.2).

A similar response was detected in pupae from 15°C adults (*P*<0.05; post-hoc Bonferroni). No response to adult acclimation was detected at any other life stage for diapause-programmed progeny. Diapause larvae from both adult temperature regimes had significantly lower SCPs than non-diapause larvae (*P*<0.001).

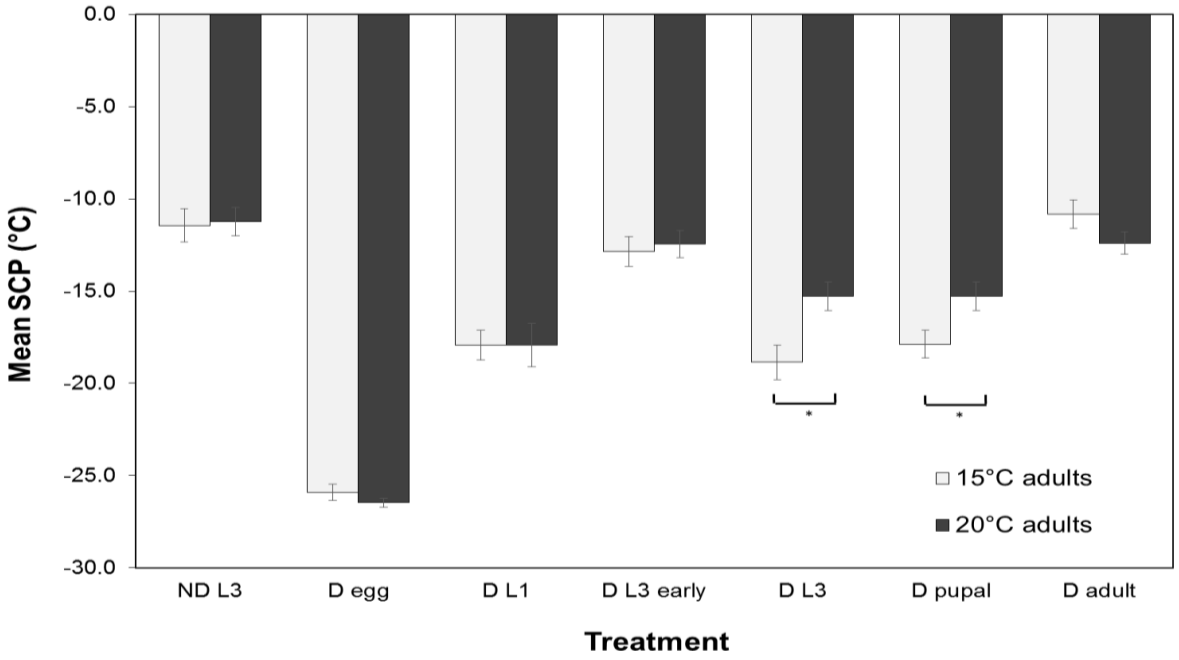

Figure 2.2 Mean SCP (± 1 S. E. M.) of *C. vicina* diapause (D) and non-diapause (ND) progeny at different stages of development from adults acclimated to either 15°C or 20°C. Means marked * are significantly different from each other for each developmental stage at (Bonferroni post-hoc). *n* = 465.

There were significant differences in the SCPs of diapause larvae produced from the cross fertilisation of parental males and females held at either 15°C or 20°C ($F_{3,91}$ = 8.0, *P*<0.001) (Figure 2.3).

The SCP was more strongly influenced by maternal than paternal acclimation temperature, with crosses involving females held at 15°C having the lowest mean SCPs. Post-hoc analysis found the SCPs of diapause larvae from ♂15° × ♀15° to be significantly lower than diapause larvae from ♂20° × ♀15° (*P*<0.05; Bonferroni post-hoc test) and ♂15° × ♀20° (*P*<0.001). The difference between the ♂15° × ♀15° cross and ♂20° × ♀20° cross was very close to significant (*P*=0.07) and was shown to be significant if using the less conservative LSD post-hoc test (*P*<0.05).

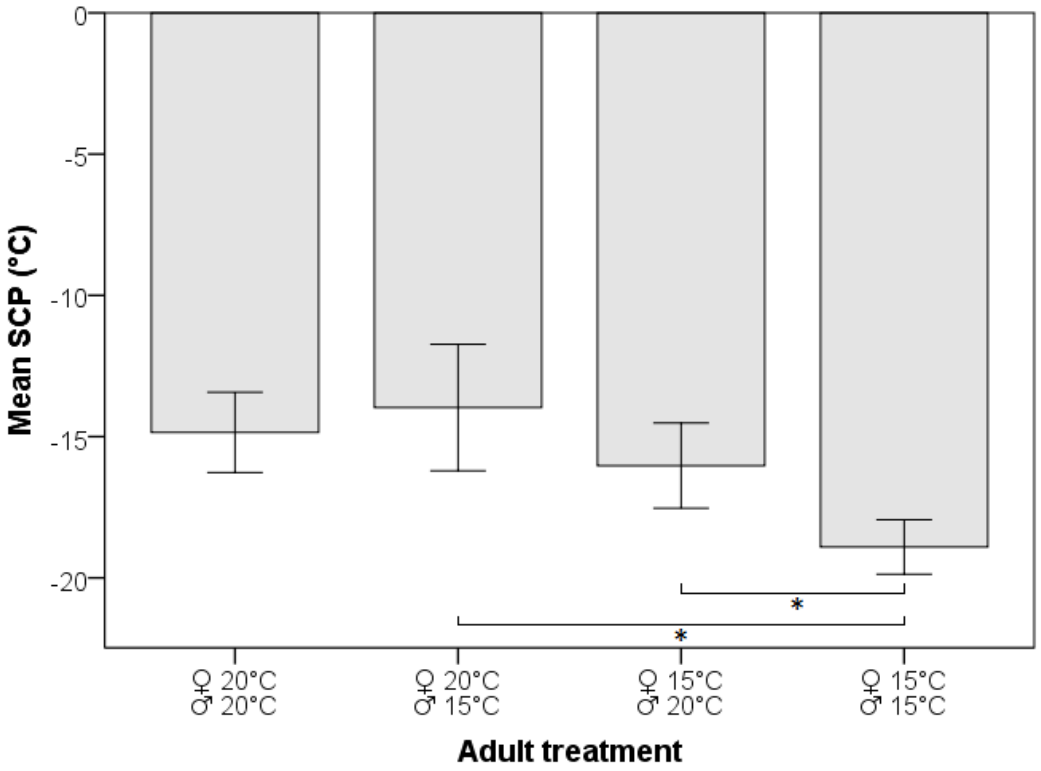

Figure 2.3 Mean SCP (± 1 S. E. M.) for *C. vicina* diapause progeny from different crosses between adult males and females separated at eclosion and acclimated to 15°C or 20°C. Means marked * are significantly different from each other for each developmental stage (Bonferroni post-hoc) (*n* = 95).

2.4.2 Lethal Times (LT)

Adult acclimation did not affect the cold hardiness of non-diapause larvae, with the exception of LT_{10} survival at -4°C (Figure 2.4a and b). Diapause larvae were significantly more cold tolerant than non-diapause larvae following both -4°C and -8°C exposure, as determined by non-overlapping fiducial limits of LT values (Figure 2.4a and b).

D15 larvae were significantly more cold tolerant than D20 larvae. This was detected following all treatments, with the exception of LT_{10} survival at -8°C (Figure 2.4a and b), and was most evident at -4°C, with a 14.6 d difference in the LT_{50} value between D15 and D20 larvae.

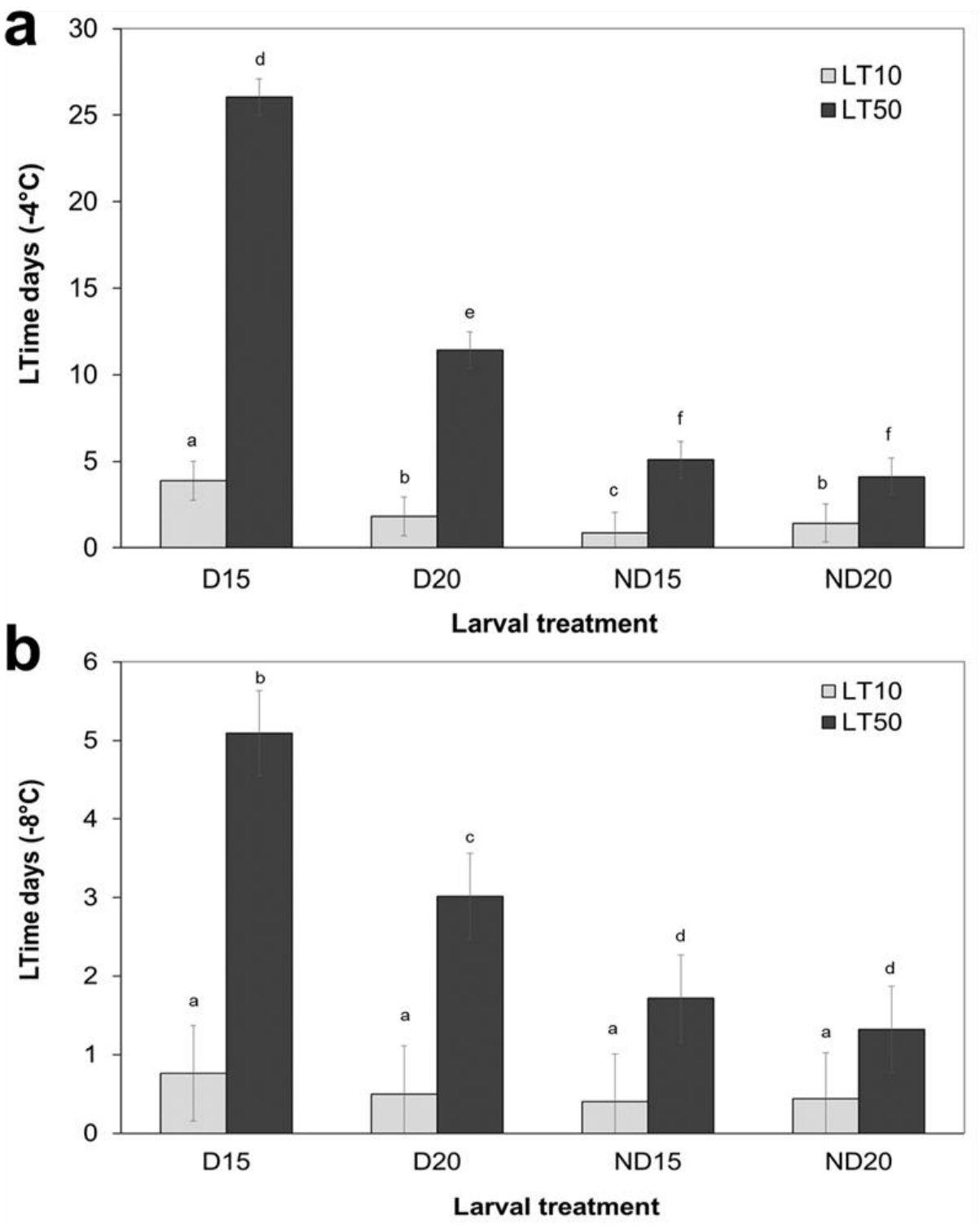

Figure 2.4 Mean Lethal Time (± 1 S. E. M.) taken to induce 10% (LT_{10}) and 50% (LT_{50}) mortality following exposure to (**a**) -4°C and (**b**) -8°C for non-diapause and diapause *C. vicina* larvae from adults acclimated to 15°C (ND15, D15) and 20°C (ND20, D20). Means marked with different letters are significantly different, as determined by non-overlapping fiducial limits.

2.4.3 Rapid Cold Hardening (RCH)

All RCH data followed that of a normal distribution ($F_{4,68}$ = 0.15, *P*=0.2). The DTs of non-diapause and diapause larvae were not significantly influenced by adult temperature ($F_{3,20}$=0.35, *P*=0.71) (Figure 2.5).

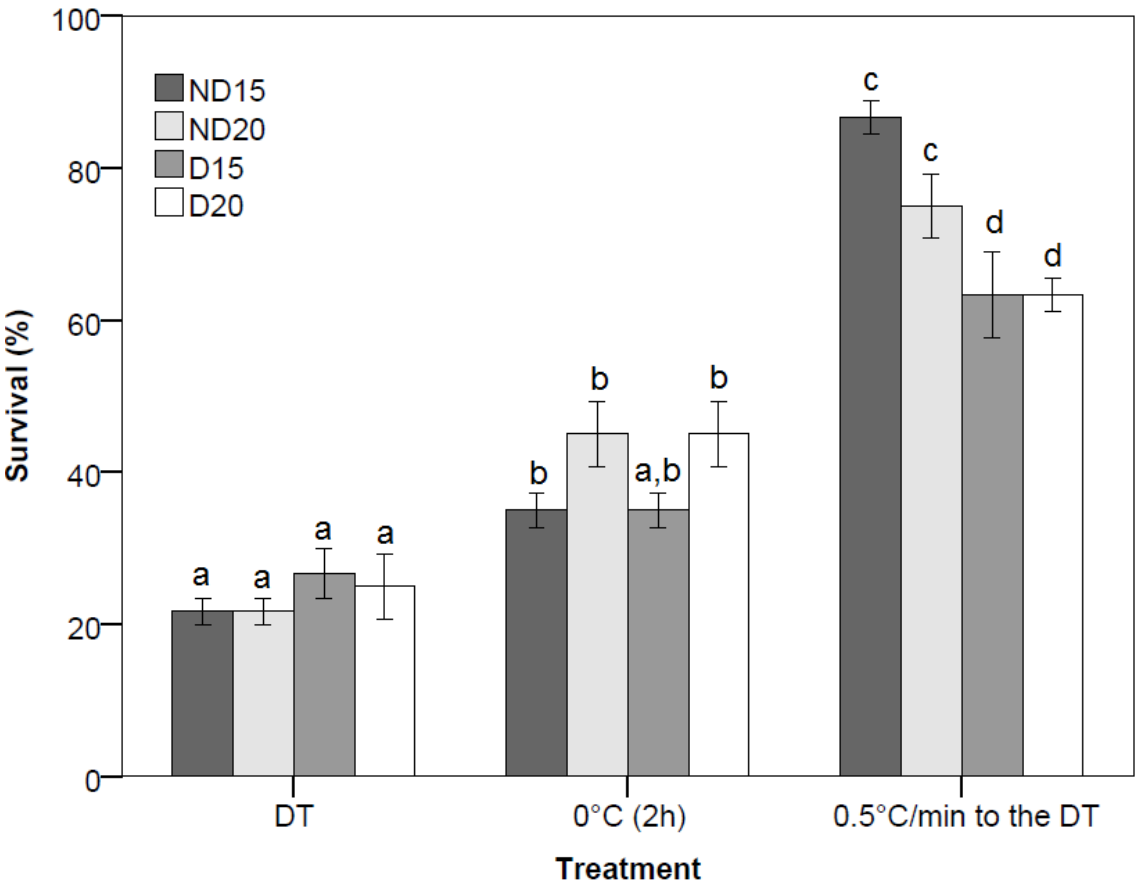

Figure 2.5 Mean survival (± 1 S. E. M.) of non-diapause and diapause *C. vicina* third instar larvae exposed directly to the discriminating temperature (DT), or following a rapid cold hardening (RCH) treatment of either 2 h at 0°C or gradual cooling at 0.5°Cmin^{-1}. Larvae from adults acclimated to 15°C (ND15, D15) and 20°C (ND20, D20). Survival was assessed as successful adult eclosion at 11°C. Means are presented (*n* = 10 × 6 replicates per data point) with those marked with different letters being significantly different from each other (Bonferroni post-hoc).

The DT for ND15 and ND20 was slightly higher (2 h at -10°C) than for D15 and D20 larvae (2 h at -11°C) although this difference was not significant (*P*=0.71).

DT survival ranged from 21.7 ± 1.7% for ND15 and ND20 larvae to 26.7 ± 3.3% for D15 larvae. A pre-treatment of 2h at 0°C prior to DT exposure was associated with a significant increase in survival relative to direct transfer to DT for ND20 (survival 45 ± 4.2%) (P<0.001), ND15 (survival 35 ± 2.2%) (P<0.001) and D20 larvae (survival 45 ± 4.3%) (P<0.05) but not for D15 larvae (survival 35 ± 2.2%) (P=0.35) (Figure 2.5). The effect of parental temperature was not significant for either diapause (P=0.07) or non-diapause (P=0.07) larvae.

Gradual cooling at a rate of 0.5°C min^{-1} to the DT induced a stronger RCH response than the constant temperature pre-treatment, and survival of all larvae was significantly greater than for their respective DT (all *P's*<0.001). There was also a significant difference in survival between non-diapause and diapause larvae ($F_{3,20}$ =8.5, P<0.001). However, post-hoc analysis did not identify a significant difference in survival associated with adult acclimation temperature for either diapause (P=0.10) or non-diapause (P=0.26) larvae.

2.4.4 Larval mass, water and glucose content

In general, wet mass was lowest for diapause larvae (Table 2.2), and significant differences were detected across the four larval treatment groups ($F_{3,76}$ = 4.9, P<0.01). ND15 larvae had the greatest wet mass, and post-hoc analysis revealed this was significantly different from D15 and D20 larvae (both *Ps*<0.05). Wet mass was not influenced by adult acclimation for either non-diapause or diapause larvae (both *P*s=1.0). Dry mass was significantly different across all treatments ($F_{3,76}$ = 4.1, P<0.01), with post-hoc analysis determining dry mass of ND15 larvae to be significantly greater than D20 larvae (P<0.05). Again, this was not influenced by adult acclimation for either non-diapause or diapause larvae (both *P's*=1.0). Water content did not vary between any larval treatment groups ($F_{3,76}$ = 2.6, P=0.6).

Glucose content was significantly different across treatment groups ($F_{3,16}$ = 28.7, P<0.001), but this was not influenced by adult culturing temperature for either diapause (P=0.3) or non-diapause (P=1.0) larvae. Overall, diapause larvae had significantly higher glucose contents than non-diapause larvae (all *Ps*<0.001).

Table 2.2 Mean (± 1 S.E.M.) and (range and DFs of) wet mass, dry mass, water content glucose content for (a) non-diapause and (b) diapause *C. vicina* larvae from adults acclimated to 15°C (ND15, D15) and 20°C (ND20, D20). Means followed by the same letter are significantly different (Bonferroni post-hoc).

	ND 15	ND 20	D 15	D 20
Wet mass (mg)	83.0 ± 2.0^{a}	83.2 ± 1.9^{b}	$74.9 \pm 1.4^{a,b}$	78.4 ± 2.0
	(65.0-97.0; n=20)	(69.0-96.0; n=20)	(63.0-86.0; n=20)	(56.0-89.0; n=20)
Dry mass (mg)	25.2 ± 0.4^{c}	24.9 ± 0.5	24.1 ± 0.4	23.4 ± 0.4^{c}
	(22.0-29.0; n=20)	(21.0-30.0; n=20)	(21.0-27.0; n=20)	(21.0-26.0; n=20)
Water content (% of dry mass)	69.5 ± 0.7	69.9 ± 0.5	67.7 ± 0.6	69.9 ± 0.8
	(60.8-72.3; n=20)	(65.7-76.0; n=20)	(61.9-73.8; n=20)	(62.5-76.0; n=20)
Glucose content ($g\ mg^{-1}$)	0.9 ± 0.2^{e}	0.6 ± 0.2^{f}	$2.4 \pm 0.2^{e,f}$	$3.0 \pm 0.3^{e,f}$
	(0.4-1.3; n=5)	(0.1-1.1; n=5)	(1.8-2.6; n=5)	2.0-3.6; n=5)

2.5 Discussion

Cross generation effects of environmental variation are well-documented in a broad range of organisms and to a wide variety of stressors (Marshall, 2008; Castro et al., 2013; Plautz et al., 2013; Suter and Widmer, 2013). Understanding these responses will be critical in determining how different organisms will cope with future climate change (Mondor et al., 2004; Rando and Verstrepen, 2007; Burgess and Marshall, 2011; Salinas and Munch, 2012). The findings of this chapter indicate that elevated temperatures experienced by a parental population of the blow fly *C. vicina*, in autumn or early winter, have the potential to reduce significantly the cold hardiness of overwintering diapause larvae in the subsequent generation. Given the widespread occurrence of a maternally regulated diapause (Mousseau and Dingle, 1991), this could be a prevalent phenomenon for insects overwintering in the temperate zone.

The cross generational influence of adult thermal history on larval cold tolerance (as determined by SCPs and LT_{50}) was not seen within the non-diapause developmental pathway, but only as part of diapause. Even within the developmental pathway of diapause programmed flies, the SCPs of eggs, L1 and even early stage L3 larvae were not affected by adult temperature. This only occurred in the third instar (L3) larval stage (i.e. at the start of

diapause; Figure 2.2). Consequently, this chapter further reinforces a direct physiological relationship between both the programming and initiation of diapause and increased cold hardiness. Increased cold tolerance has been associated with diapause in a number of species (such as Kostál and Simek, 2000; Goto et al., 2001; Vesala and Hoikkala, 2011) and some of the underlying cold tolerance mechanisms are well understood (Denlinger and Lee, 2010; Khodayari et al., 2013). However, the mechanisms linking the photoperiodic programming of diapause with variation in temperature, and the subsequent cross generational control over cold tolerance phenotypes in the next generation remain an unknown. The evidence from male × female crosses held at different temperatures (Figure 2.3) indicates that it is the thermal history of the mother that has the greatest influence over diapause-associated cold tolerance. This is in agreement with previous studies in *C. vicina* where both diapause incidence and duration phenotypes are mainly controlled by the maternal line (McWatters and Saunders, 1998). This phenomenon has also been identified in other species, such as the mosquito, *Aedes togoi* (Kappus and Venard, 1967) and the parasitic wasp, *Trichogramma evanescens* (Vinogradova and Zinovjeva, 1972).

Diapausing *C. vicina* exhibited a stronger supercooling capacity compared to non-diapause larvae irrespective of parental temperature conditions (Figure 2.2) and the lowest SCPs were associated with a lower parental acclimation temperature. It is important to recognise that SCP temperatures represent the physiological limit of supercooling, and not the ecological limit of survival (Bale, 1996; Renault et al., 2002). However, a number of studies have identified a correlation between supercooling capacity and low temperature tolerance, including for the Heteroptera, *Pyrrhocoris apterus* (Hodkova and Hodek, 1997) and the Coleoptera, *Coccinella septempunctata* and *Semiadalia undecimnotata* (Kalushkov and Nedved, 2000). For this reason the SCP continues to be used to provide insight into the physiology of cold hardiness and as a useful comparative index (Renault et al., 2002). As found in previous studies (e.g. Saunders and Hayward, 1998; Johnson, 2013), diapause larvae were also significantly more cold tolerant than non-diapause larvae (Figure 2.4a and

b). Parental thermal history only had an effect within the diapause programme, and differences in cold tolerance were greatest between D15 and D20 larvae following exposure to -4°C, with a 15 d difference in LT_{50} values. The causes of mortality at sub-zero temperatures include cellular damage, enzyme blockage and the accumulation of metabolites which become toxic at high concentrations (Renault et al., 2002). Protective mechanisms against these damaging effects are a well-known part of diapause (Hahn and Denlinger, 2007; Rinehart et al., 2007; Rozsypal et al., 2013; Teets and Denlinger, 2013), and the data presented in this chapter suggest that the cross generational programming of at least some of these stress tolerance mechanisms is influenced by the thermal, and not just the photoperiodic, history of the parental generation.

Despite these differences in chronic cold tolerance, there were no differences in survival following acute cold stress between diapause and non-diapause larvae, as determined by the DT (Figure 2.5). Both non-diapause and diapause larvae demonstrated RCH, with a slight increase in survival following 2 h at 0°C, and a significant increase in survival following gradual cooling to DT at 0.5°C min^{-1} (Figure 2.5). The strongest RCH response was seen in non-diapause larvae, which supports the idea of diapause being an anticipatory response to chronic winter cold (Tauber et al., 1986) rather than an immediate response to extreme and acute cold shock. For diapausing *C. vicina* in particular, the winter period is spent in the thermally buffered soil microhabitat (Saunders and Hayward, 1998), where selective pressures to enhance tolerance to prolonged cold exposure are stronger than evolutionary pressures to tolerate sudden temperature fluctuations (Saunders, 1987; Vaz Nunes and Saunders, 1989). Adult acclimation had no effect on the RCH ability of diapause or non-diapause larvae following 2 h exposure to 0°C prior to DT exposure, or gradual cooling to the DT. However, non-diapause larvae from 15°C adults showed a markedly stronger RCH response (86.7 ± 2.1%) than non-diapause larvae from 20°C parents (75.0 ± 4.3%) (Figure 2.5). While this difference was not significant, it may warrant further investigation, and would

represent a cross generation control of cold tolerance that was not linked to the diapause programme.

Previous work on *C. vicina* has identified glucose as the dominant compound in all non-diapause life stages (Block et al., 1990). However, whether levels of glucose change as part of the diapause programme has never been investigated in this species. Increased levels of glucose have been strongly associated with diapause in several other insect species (Pullin and Wolda, 1993; Overgaard et al., 2007; Michaud et al., 2008; Hou et al., 2009; Ragland et al., 2010; Xu et al., 2012), with a hypothesized role in it enhancing cold tolerance. There was a three-fold increase in glucose concentration in *C. vicina* diapause larvae (Table 2.2), which combined with greater cold tolerance (Figure 2.4) and lower SCPs (Figure 2.2), provide further correlative evidence for glucose potentially contributing to increased cold hardiness. However, there was a slight reduction (although not significant) in glucose levels between D20 and D15 larvae, despite the latter being significantly more cold tolerant and having a lower SCP. Thus, it seems glucose may not contribute directly to cold tolerance in this instance, but could instead be being metabolised into other cryoprotectants, such as trehalose and glycogen, in D15 larvae both compounds being associated with enhanced cold tolerance (Storey, 1997; Arrese and Soulages, 2010). The specific role of glucose clearly requires further investigation, although it has been associated with both diapause and RCH in previous studies (Michaud and Denlinger, 2007; Overgaard et al., 2007; Michaud et al., 2008; Hou et al., 2009). However it is unclear whether up-regulation is due to a role in cold protection or as a by-product of glycolysis (Michaud and Denlinger, 2007).

Phenotypic plasticity has been described as a bet-hedging strategy to enhance survival in an unpredictable environment (Simons and Johnston, 1997). Both costs and benefits are associated with acclimation (Huey et al., 1999), although the species most at threat from climate change are those in which evolutionary and plastic adjustments are either impossible or slower than changing environments (Chown et al., 2010). The phenotypic adjustment identified here would prove beneficial if higher adult temperatures in autumn were indicative

of warmer conditions for larval progeny during winter (as suggested by the beneficial acclimation hypothesis) (Wilson and Franklin, 2002). However, climate models predict that while autumns will continue to grow warmer (IPCC, 2013), the winter will continue to experience periods of prolonged cold. Some recent studies even suggest that as Arctic ice sheets continue to melt, winters will become longer and colder across Europe and North America (Jaiser et al., 2012). This chapter has presented a previously unrecognised threat - parental thermal history has a cross generational effect on the cold tolerance of their diapausing progeny.

2.6 Conclusions

Warmer autumn conditions will disrupt the diapause programme in a number of ways. Higher temperatures will cause sensitive stages to develop faster, providing less time to detect photosensitive cues (Mousseau and Dingle, 1991) and therefore produce a weakened diapause induction (Saunders, 1987), which may result in diapause being averted completely or ending before the end of winter (Bale and Hayward, 2010). This chapter identified an additional, and previously unrecognised, threat that higher temperatures experienced by the maternal generation, although not sufficiently high to abort diapause, will reduce the cold tolerance ability of diapausing larvae in the subsequent generation. All scenarios could result in a significant increase in winter mortality. As a successful ubiquitous species, it is unlikely that the long-term survival of *C. vicina* is under threat, although its distribution range at high latitudes may be affected. However, given that maternal regulation of diapause is common among temperate insects (Mousseau and Dingle, 1991), this response could impact a wide range of insect species.

This chapter has focused on the ability of diapause and non-diapause life stages to tolerate chronic cold exposure. The winter is a period of both prolonged cold and frequent temperature fluctuations, and for this reason it is also of important to consider acute cold

tolerance. The acute cold tolerance of diapause and non-diapause life stages are investigated in detail in the following chapter.

CHAPTER 3

DIAPAUSE AND DIET INFLUENCE RAPID COLD HARDENING AND ACTIVITY THRESHOLDS OF *CALLIPHORA VICINA*

3.1 Abstract

The timing of diapause is being disrupted for many temperate insects due to warmer autumn conditions. For the blow fly, *Calliphora vicina*, observational evidence has shown that larvae are averting diapause, leaving non-diapause larvae and adults exposed to acute cold. This chapter assess the ability of non-diapause and diapause larvae, fed and unfed adults to enhance survival through rapid cold hardening (RCH). In addition, the lower thermal activity thresholds of adults (CT_{min} and chill coma) are identified, to determine when activity may be possible during the winter.

RCH was determined as the ability to heighten survival at a discriminating treatment (temperature inducing 20% survival) through prior exposure to a less severe temperature. Non-diapause larvae exhibited the strongest response, followed by unfed adults, and diapause larvae. Fed adults displayed no RCH response. Adult feeding had a similar effect on activity thresholds; fed adults entered chill coma at higher sub-zero temperatures than unfed adults.

These findings indicate that, following diapause aversion, temperatures will remain sufficiently high for non-diapause larvae to survive the winter. For adults, survival will be influenced by access to food. Temperatures regularly fall below those shown to impair movement and cause mortality of fed adults. Survival of unfed adults will be enhanced through the presence of a RCH response and lower activity thresholds.

3.2 Introduction

Within the temperate zone, there is a close association between the life history of the local fauna and flora and the changing seasons. This association is noticeable in the pre-emptive ability of many species to prepare for winter, either through migration, or implementing behavioural and physiological modifications *in-situ* (Tauber et al., 1986). These responses are induced through detection of changing environmental stimuli such as lower temperatures

or shortened photoperiods. An important *in-situ* adaptation associated with overwintering temperate insects is that of diapause (Denlinger, 2002).

For the southern green stink bug, *Nezara viridula*, the warmer winters in the tropics are alleviating the cold-induced stress experienced by diapause adults, resulting in a shift in the northern limit of its range, particularly in central Japan (Musolin, 2012). In addition, climate change is positively affecting other species through an extension of the growing season. Longer summers and a delay in the timing of diapause potentially allow the inclusion of additional yearly generation in multivoltine species, as long as sufficient day degrees are acquired (Bale and Hayward, 2010). However, not all species are responding positively to climate change. Uncharacteristically high late-winter temperatures in the Swiss Alps between 1989 and 1991 instigated an early breaking of diapause in the larch bud moth, *Zeiraphera diniana* (Emelianov et al., 1995). This was followed by a return to lower temperatures, causing high levels of mortality and a crash in the population. Warmer winters are also associated with heightened mortality in overwintering larvae of the endangered glacial butterfly, *Boloria eunomia*, and adults of the solitary bee, *Osmia lignaria*, due to increased disease and fungal outbreaks (Radchuk et al., 2013) and depletion of energetic reserves (Sgolastra et al., 2011). Another consequence of warmer autumns is that energy reserves laid down by the insect to survive winter are being utilised before spring, resulting in winter starvation or greatly reduced fitness at spring emergence (Williams et al., 2012).

In the blow fly *Calliphora vicina*, as with many insects, diapause is induced following maternal detection of decreasing photoperiods. Previous fieldwork has identified that the Birmingham (52.4°N, 1.9°W) strain of *C. vicina* has a CDL, inducing 50% diapause incidence, of 14.5 h. This day length (sunrise to sunset) is experienced around the 11th August (at 52.4°N, 1.9°W), but, it is not known if *C. vicina* are sensitive to the low levels of light experienced during twilight (dawn and dusk), or if high levels of light sensitivity are required to induce diapause (Johnson, 2013) . If *C. vicina* are not sensitive to low levels of light it is possible that diapause induction occurs slightly later in the year (Johnson, 2013),

but egg oviposition collected from adult cultures established in the field on 14th September 2009 showed a 79% incidence of diapause on the 28th October (Johnson, 2013). It can therefore be assumed that sufficient diapause inducing CDLs are being experienced by mid-September for *C. vicina* populations in the wild.

Provided temperatures remain below critical thresholds , diapause is experienced by third-instar larvae of the subsequent generation (Saunders, 1987; Saunders and Hayward, 1998). However, if temperatures exceed 20°C for adults, or 15°C for larvae, this ability to enter diapause is aborted and the non-diapause developmental pathway ensues. Following diapause aversion, if insufficient day degrees are experienced to allow completion of another life cycle, and thus later entry into diapause, individuals risk any stage of their life cycle being exposed to winter conditions (Bale and Hayward, 2010). In addition to chronic low temperature exposure, life stages outside of diapause are increasingly likely to encounter more severe and acute temperature fluctuations over diurnal time-scales, as both non-diapause larvae and adults may be active above ground, compared to diapause larvae which burrow into the soil. It is possible that non-diapause larvae and adults have the ability to acquire protection against acute cold-stress through the process of RCH, a response which has been shown to provide cold protection in species that naturally lack overwintering strategies, such as the monarch butterfly, *Danaus plexippus*, and the sycamore lace bug, *Corythucha ciliata* (Larsen and Lee, 1994; Ju et al., 2011). To date, the RCH ability of any life stage of *C. vicina* has not been assessed.

RCH is the ability to enhance survival at extreme low temperatures through prior exposure, of minutes to hours, to a less severe temperature (Bale, 2002; Lee et al., 2006a). First identified in the flesh fly, *Sarcophaga crassipalpis* (Lee et al., 1987), it has since been reported in a range of organisms (Terblanche et al., 2008; Nyamukondiwa and Terblanche, 2010; Everatt et al., 2012), although is notably absent from some species (Sinclair et al., 2003). Unlike diapause, it is a short-term response, occurring during any life stage and at any time of the year.

Despite the different time scales involved, some of the biochemical and physiological mechanisms associated with RCH are similar to those induced during diapause. These include the synthesis of low molecular weight polyols and amino acids (Koštál and Simek, 2000; Overgaard et al., 2007), the up-regulation of HSPs (Rinehart et al., 2007) and a lowering of the SCP (Bale et al., 1984). However, work on *S. crassipalpis* and *Drosophila melanogaster* has recognised that these responses play only a minor role in RCH (Kelty and Lee, 1999; Lee et al., 2006a). Instead, RCH is mainly associated with the blocking of cold-induced apoptosis through inhibition of specific caspases responsible for cell death (Yi and Lee, 2011). Although the physiological changes underpinning RCH are increasingly well understood, relatively little is known about the role RCH may play in winter survival of species where diapause is averted as a result of warmer autumn conditions. Winter survival will be dependent upon the proximity of conditions encountered to the physiological limits of survival (Chown et al., 2010; Hoffmann, 2010). This chapter seeks to determine if these limits can be extended through the process of RCH.

Another response to temperature fluctuations is the loss of coordinated movement as temperatures decrease (Renault et al., 1999). As insects are cooled they pass through a conserved set of physiological responses. At first, activity becomes impeded and walking speed decreases until the ability to maintain coordinated movement is lost - the critical thermal minimum (CT_{min}). Beyond this point there is continued appendage movement until the individual reaches a point of chill coma, a reversible physiological state signified in this chapter by a final appendage twitch (Hazell and Bale 2010). As with cold tolerance, the temperature at which insects reach these activity thresholds can be lowered through acclimation (Rako and Hoffmann, 2006). For motile life stages, these activity thresholds may be of more ecological relevance than the lower limits of survival (Coombs and Bale, 2013). For example, at temperatures below the CT_{min} the individual is unable to locate food, disperse to more favourable locations, reproduce or escape predation (Alford et al., 2012; Everatt et al., 2013). Consequently, these activity thresholds are of importance when

predicting insect distributions and range shifts in response to climate change (Hazell et al., 2010).

This chapter compares the RCH ability of non-diapause and diapause *C. vicina* larvae as well as adults, in order to determine whether a RCH ability has the potential to heighten winter survival. This chapter also assesses how cooling rate influences the extent of RCH, the lower lethal temperature (which is the lowest temperature at which survival is possible) and the SCP. In addition, the lower thermal activity thresholds of *C. vicina* adults, and the impact that protein feeding has on activity threshold and RCH is investigated. These findings are discussed in the context of how disruption of diapause by climate change might influence winter survival and life-history strategies.

3.3 Materials and Methods

3.3.1 Experimental cultures

C. vicina used in this chapter were originally sourced from the University of Birmingham campus, Birmingham, UK (52.4°N, 1.9°W) in 2009 using olfactory-traps (Hwang and Turner, 2005) (Figure 2.1) and lab cultures were then regularly replenished with wild caught individuals.

For culturing of experimental flies and the production of non-diapause and diapause larvae please refer to Chapter 2, Section 2.3.2. All adult cultures were maintained at 20°C under either a long-day (LD 18:6 h) or short-day (LD 12:12 h) photoperiod. Larvae from the short-day parents were predominantly diapause and those from long-day parents predominantly non-diapause. Diapause and non-diapause larvae are morphologically indistinguishable, but diapause larvae can be identified as larvae not pupariating by d 30 post-oviposition (Richard and Saunders, 1987). For this reason, non-diapause larvae were used for experimental use on d 15 post oviposition, while diapause larvae were used d 30.

3.3.2 The discriminating temperature (DT)

The discriminating temperature (DT) is determined as that giving rise to approximately 80% mortality following a 2 h exposure period (Lee et al., 1987). This was determined following the plunge method (Nunamaker, 1993). Individuals were placed in 50 ml test tubes and plunged into an alcohol bath (Grant LTD D6C, Grant Instruments, UK) at 1°C increments from -4°C to -18°C for 2 h. For non-diapause and diapause larvae survival was measured as successful development to the adult life stage at 11°C. Six replicates of n = 10 were assessed for each treatment group.

The ability to undergo RCH was assessed in both diapause and non-diapause larvae (6 × 10 replicates) under the following treatments:

- 2 h at -1, -2 or -5°C, before exposure to DT,
- 1 h at 0°C then 1 h at -5°C before DT,
- gradual cooling at 0.1, 0.5 or 1°C min^{-1} to DT,
- gradual cooling at 0.1 or 0.5°C min^{-1} to 0°C before DT.

3.3.3 Effect of RCH on larval supercooling capacity

The effect of RCH on the SCP of diapause and non-diapause larvae (minimum of n = 22 for each treatment) was determined by attaching individual larvae to type K exposed wire thermocouples using a small amount of adhesive OecoTak A5® glue (Oecos Ltd, Kimpton, Hertfordshire, UK). Individual larvae were then placed into 1 ml Eppendorf tubes® (Sigma-Aldrich, Gillingham, Dorset, UK), which were placed in boiling tubes submerged in a programmable alcohol bath (two tubes per boiling tube) (Bale et al., 1984). The temperature was reduced at 0.1°C, 0.5°C or 1°C min^{-1} from 11°C to -30°C and the SCP detected by an exothermic output upon freezing (Figure 1.6).

3.3.4 Limits of the larval RCH response

The lower limit of temperature tolerance following RCH was determined by gradually cooling non-diapause larvae at a rate of 0.5°C min^{-1} to temperatures below the DT (at 1°C increments) for 2 h, followed by transfer to 11°C and survival assessed as successful eclosion.

3.3.5 Adult RCH

DT was assessed as described for larvae and survival assessed as spontaneous movement after 2 h recovery at 20°C. All adults were maintained under LD 12:12 h at 20°C. The effect of providing a protein source on RCH was determined by either providing adult flies with free access to liver/protein on d 4 or 6 (PF) or excluding liver/protein (PE). All flies still received sugar and water *ad libitum* and so were not starved or dehydrated. RCH was assessed in PF and PE (6 × 10 replicates) under the following treatments:

- 3 h at 0°C, 5°C or 10°C, before exposure to DT,
- after being maintained under an ecologically relevant thermoperiod cycle for 6 d. The thermoperiod was typical of late-autumn temperatures experienced by adult *C. vicina* in Birmingham, UK. A programmable incubator (LD 12:12 h) cycled from 10°C to 20°C and back to 10°C over a 24-h period (thus approximately 1.25°C h^{-1}). Adults were established under these conditions at 10°C (00.00h) and removed during the warming phase at 10°C, 15°C and 20°C on d 6.

3.3.6 CT_{min} and chill coma of adults

The CT_{min} and chill coma of adult flies were assessed following the methods of Hazell et al. (2008). Adults were placed in a central cooling arena (40 mm in diameter × 7.5 mm in depth) that allowed passage of cooled fluids from an attached alcohol bath (Haake Phoenix 11 P2, Thermo Electron Corp., Karlsruhe, Germany). Adults were prevented from escaping by

covering with a microscope slide (76 × 26 mm). A type K thermocouple connected to a thermometer (Tecpel Advanced Digital Thermometer DTM-315, Heatmiser, UK) was inserted into the arena wall, which recorded the temperature throughout the experiment. The cooling arena is illustrated in Figure 3.1.

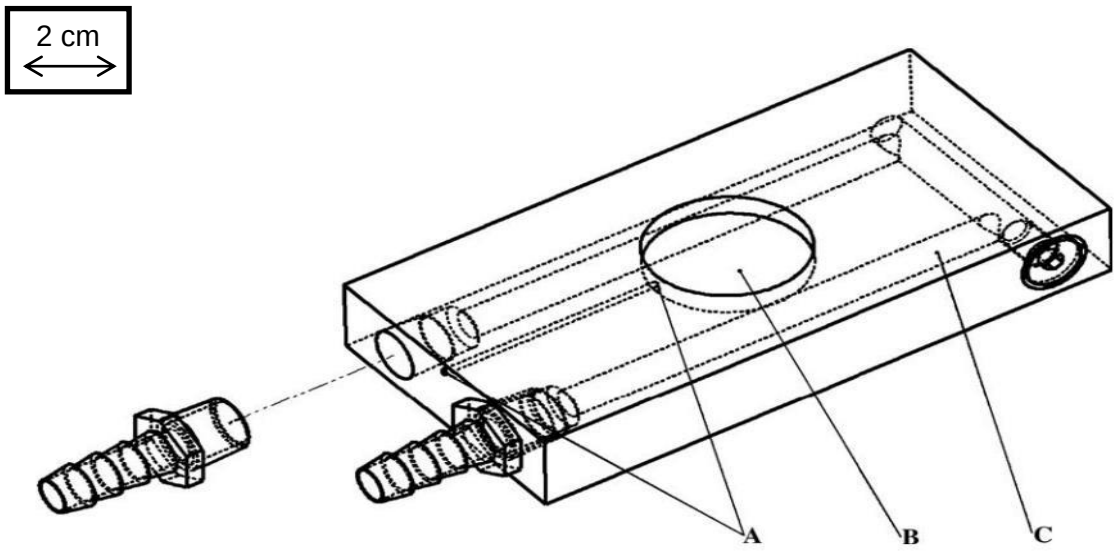

Figure 3.1 Design of the temperature controlled arena for measuring activity thresholds: A is the location of thermocouple; B is the central arena for the insect: and C is the channel for the circulation of temperature controlled alcohol (source: Hazell et al., 2008).

Activity was recorded using a digital camera (Infinity 1-1, Lumenera Scientific, Ottawa, Canada) and macro-lens (Computer MLH-10X, CBC Corp., USA), and video recording software (Studio Capture DT, Studio 86 Designs, Lutterworth, UK).

The effect of age on CT_{min} and chill coma was determined by using flies on d 0, 4 and 6 post-eclosion, for PF and PE adults. A minimum of 13 individuals were used for each treatment.

3.3.7 The supercooling capacity of adults

Adult SCPs were recorded on d 0, 4 and 6 post-eclosion for both PF and PE treatments. This followed the same procedure as outlined for larvae. Individuals were cooled at 0.5°C min^{-1} (minimum of 8 × 3 replicates for each treatment).

3.3.8 Statistical analysis

Lower thermal activity thresholds had to be calibrated against direct temperature recordings from immobile adults held within the cooling arena. Calibrated data were then subjected to a Kolmogorov-Smirnov test to identify the distribution that best described results. Activity threshold, RCH and SCP were analysed using separate GLMs in SPSS (v. 20.0, IBM, New York, USA) and significant differences between groups identified using Tukey's multiple range test with the alpha threshold set at 0.05.

3.4 Results

3.4.1 Determination of the DT in larvae

Larval survival decreased as they were exposed to lower temperatures (Figure 3.2).

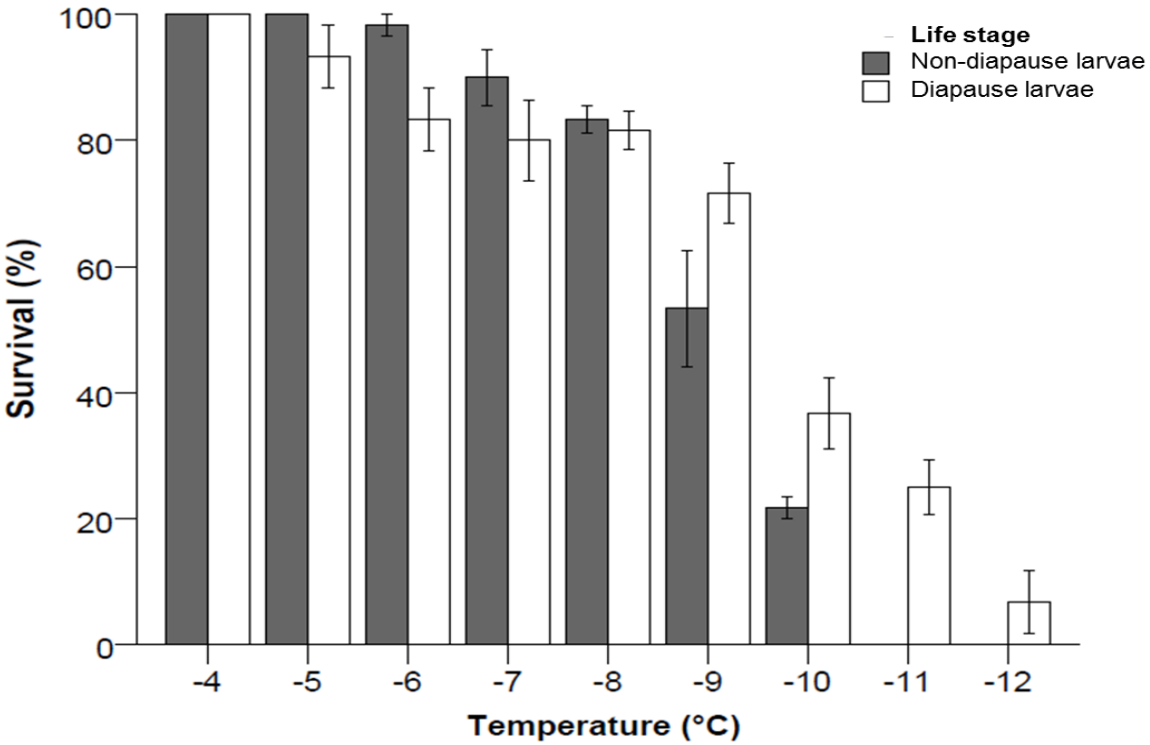

Figure 3.2 Mean survival (± 1 S.E.M) of *Calliphora vicina* diapause larvae and non-diapause larvae following 2 h exposure to progressively lower sub-zero temperatures (-4° to -12°C). Survival was assessed as successful adult eclosion at 11°C for larvae and spontaneous movement after 2 h recovery at 20°C for adults. Means are presented. *n* = 6 × 10 replicates for each bar.

Diapause larvae displayed greatest cold tolerance, with a DT of 2 h at -11°C (25 ± 4.3% survival), compared to DT of 2 h at -10°C for non-diapause larvae. However, the overall survival of diapause and non-diapause larvae were not significantly different ($F_{1,90}$ = 0.22; *P*=0.65).

3.4.2 Larval RCH response

3.4.2.1 Direct transfer

RCH data following direct transfer were normally distributed (F_{25} = 0.18; *P*=0.19). Both non-diapause and diapause larvae significantly increased survival at the DT following constant temperature RCH pre-treatments ($F_{4,25}$ = 26.30; *P*<0.001 and $F_{5,25}$ = 8.54; *P*<0.001, respectively) (Figure 3.3).

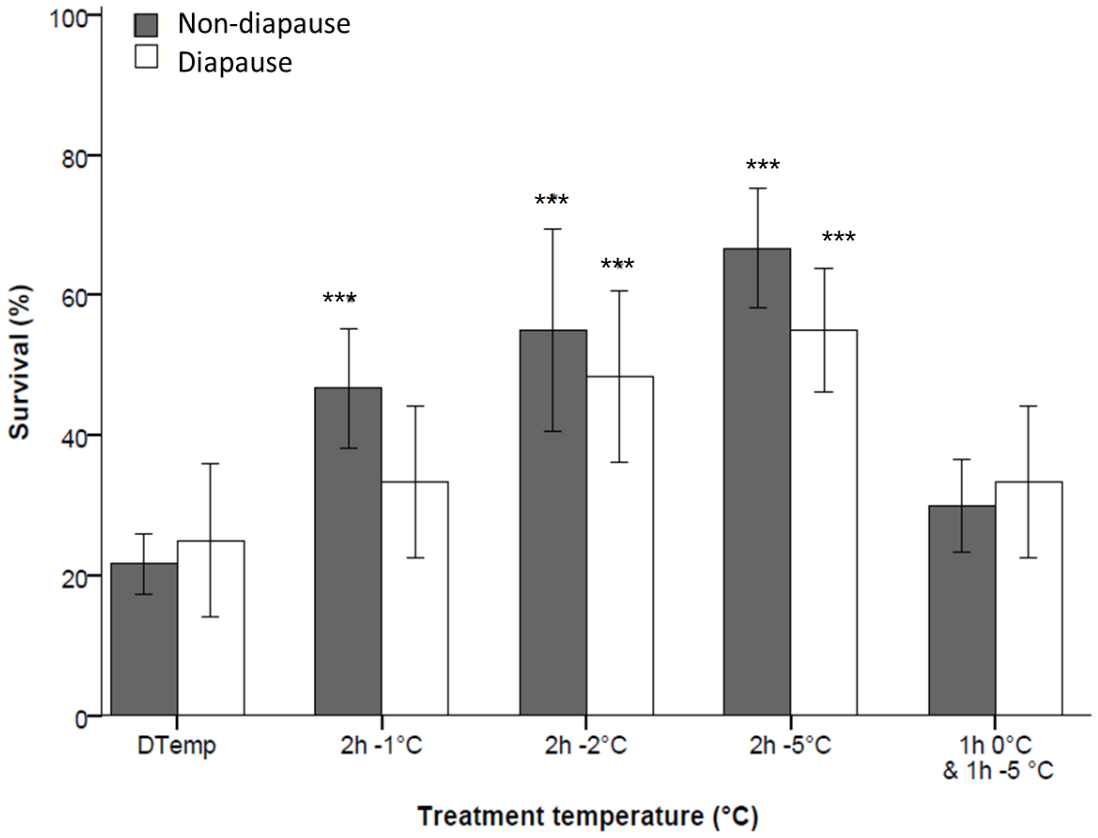

Figure 3.3 Mean survival (± 1 S. E. M.) as a measure of Rapid Cold Hardening (RCH) ability of non-diapause and diapause *Calliphora vicina* larvae following 2 h constant temperature pre-treatments, *n* = 6 × 10 replicates per data point. *** Significantly different from survival following direct transfer (DTemp) for the same treatment group.

For non-diapause larvae, survival at the DT increased significantly following 2 h at -1°C (46.7 ± 3.3%), -2°C (55.0 ± 5.6%;) and -5°C (66.7 ± 3.3%) (all *P*s<0.001; Tukey tests). Diapause larvae pre-treated at -1°C did not demonstrate a strong RCH response, and survival was not significantly different from direct transfer to DT (*P*=1.0; Tukey test) (Figure 3.3). However, a RCH response was observed for diapause larvae following 2 h at -2°C (48.3 ± 4.7%) and at -5°C (55.0 ± 3.4%) (both *P*s<0.001; Tukey tests). For both non-diapause and diapause larvae, the combined treatment of 1 h at 0°C followed by 1 h at -5°C failed to significantly increase survival beyond direct transfer to the DT (30.0 ± 2.6%; *P*=1.0 and 33.3 ± 4.2%; *P*=1.0, respectively). Overall, within-group differences between survival of non-diapause and diapause larvae were not significant (all *Ps* >0.25).

3.4.2.2 Gradual cooling

Gradual cooling of non-diapause larvae also significantly increased survival compared to direct transfer to DT ($F_{5,54}$ = 16.77, *P*<0.001) (Figure 3.4).

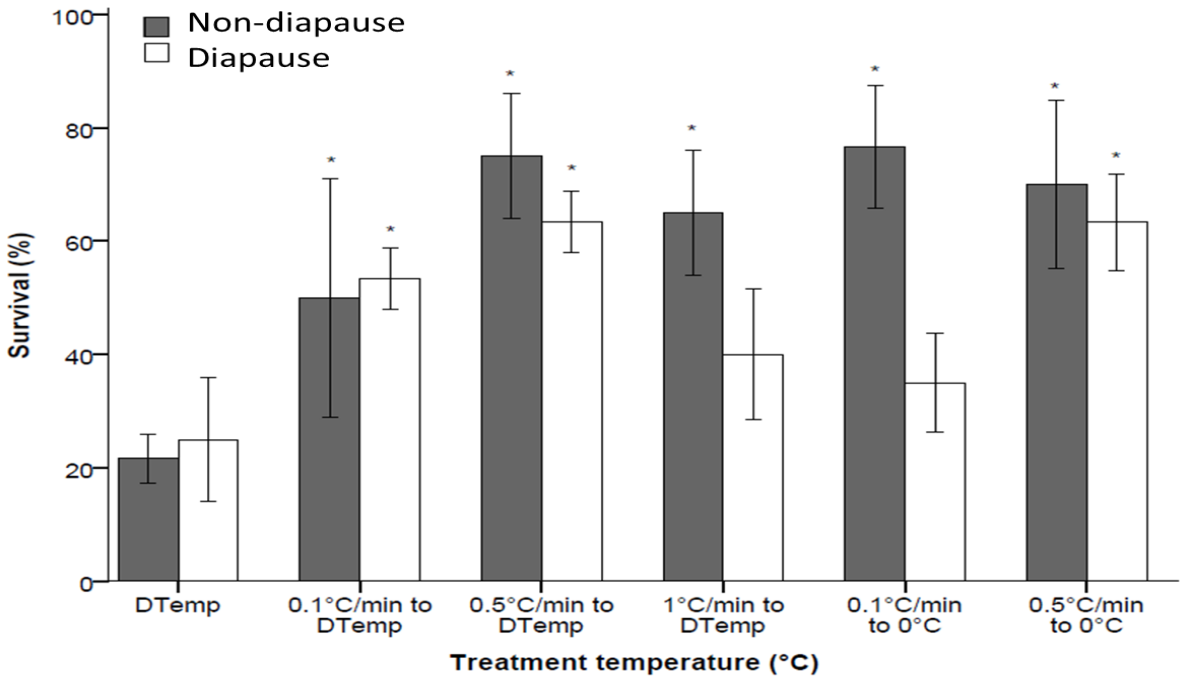

Figure 3.4 Mean survival (± 1 S. E. M.) as a measure of Rapid Cold Hardening (RCH) ability of non-diapause and diapause *Calliphora vicina* larvae following gradual cooling at different rates. Survival was assessed as successful adult eclosion at 11°C. *n* = 6 × 10 replicates per data point. * significantly different from DTemp for the same treatment group.

The greatest RCH response seen at a ramping rate of 0.5°C min^{-1} or following cooling to 0°C at 0.1°C min^{-1} prior to transfer to DT. All other cooling regimes also significantly increased survival of non-diapause larvae at DT (all *P*s<0.01). Diapause larvae displayed a weaker RCH response, although survival increased significantly following the majority of cooling rates ($F_{5,54}$ = 21.42, *P*<0.001), with the exception of 1°C min^{-1} to DT and cooling to 0°C at 0.1°C min^{-1} prior to transfer to DT (*P*s=0.06 and 0.71, respectively).

3.4.3 The effect of RCH on larval SCPs

Cooling rate had no significant effect on the supercooling capacity of non-diapause larvae ($F_{2,75}$ = 0.89, *P*=0.4) (Table 3.1).

Table 3.1 Mean supercooling point (SCP) (± 1 S.E.M) for diapause and non-diapause *Calliphora vicina* under different cooling rates. *n* = 22 to 62 replicates per data point (provided in brackets).

Cooling rate (°C min^{-1})	Diapause larvae (*n*)	Non-diapause larvae (*n*)
1	-13.7 ± 1.0°C (24)	-11.9 ± 1.5°C (28)
0.5	-18.9 ± 0.5°C (62) **a**	-11.4 ± 0.9°C (28)
0.1	-15.7 ± 0.8°C (27)	-12.8 ± 0.9°C (22)

Means followed by 'a' are significantly different from other means in the same column (Tukey's HSD, *P*>0.05)

However, cooling rate had a significant effect on the SCP of diapause larvae ($F_{2,110}$ = 23.8; *P*<0.001), with those cooled at 0.5°C min^{-1} having a significantly lower SCP than diapause larvae cooled at 1°C min^{-1} and 0.1°C min^{-1} (*P*<0.05; Tukey test) (Table 3.1).

3.4.4 Limits of the larval RCH response

Survival of non-diapause larvae following gradual cooling to -11°C , -12°C and -13°C was significantly higher than survival following direct transfer to DT (2 h at -10°C) (all *P*s<0.05; Tukey tests). The lower lethal temperature of non-diapause larvae following cooling at 0.5°C min^{-1} was lowered to 2 h at -16°C (Figure 3.5), compared to 0% survival after 2h at -11°C following direct transfer (Figure 3.2).

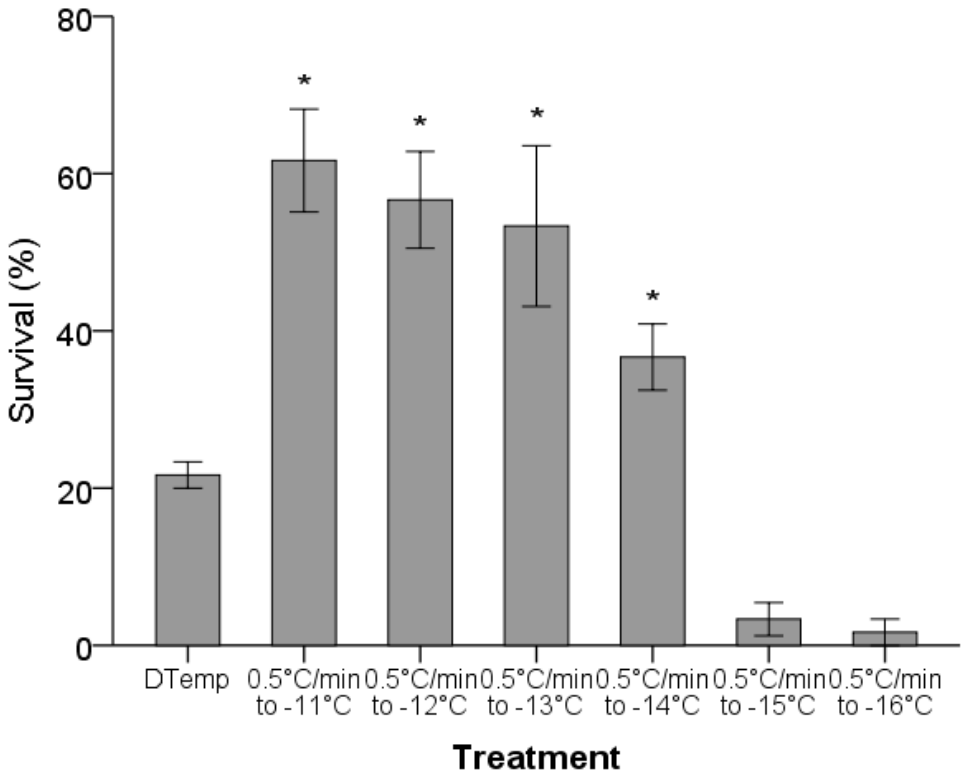

Figure 3.5 Mean survival (± 1 S. E. M.) of *Calliphora vicina* non-diapause larvae following gradual cooling at 0.5°C min^{-1} to progressively lower sub-zero temperatures (-11° to -16°C). Survival was assessed as successful adult eclosion at 11°C. Means based on with 6 × 10 replicates per data point. * significantly different from survival at the discriminating temperature (DT) for the same treatment.

3.4.5 Determination of the DT in adults

Adults provided with liver (PF) were not as cold tolerant as flies without access to protein (PE) (Figure 3.6). However, survival was not significantly different between treatments (*P*=0.18; Tukey test). DTs were determined as 2 h at -8°C for PE adults and 2 h at -7°C for PF adults.

3.4.6 Adult RCH

PE adults displayed a strong RCH response following pre-treatments at 0°C, 5°C and 10°C (Figure 3.7), with significantly higher survival than direct transfer to the DT ($F_{6,84}$ = 1.25, *P*<0.05).

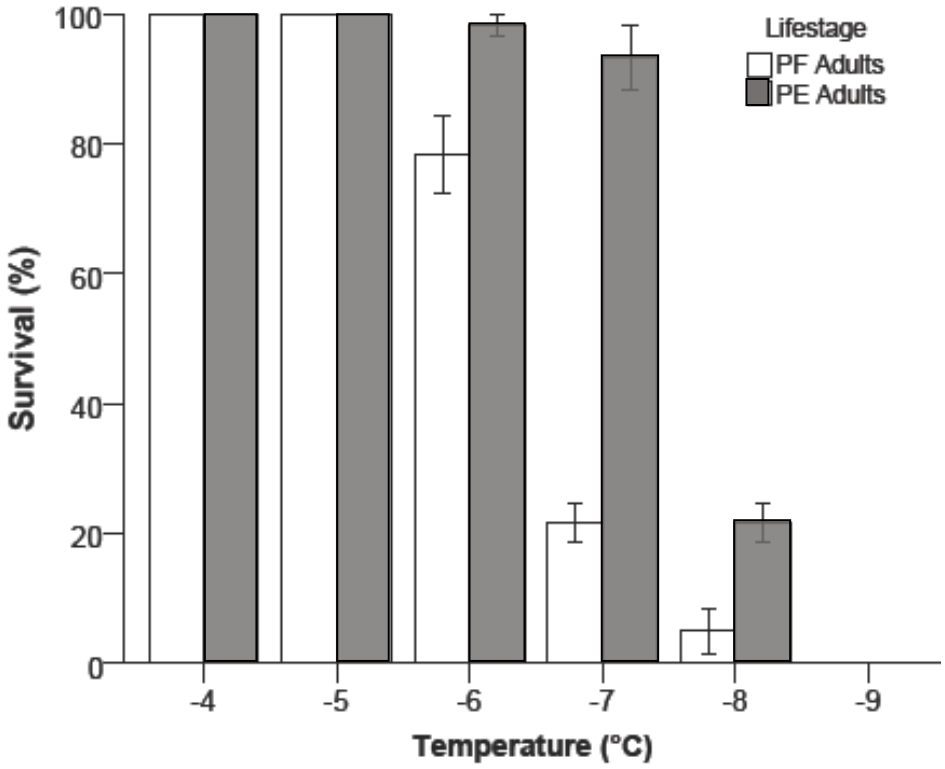

Figure 3.6 Mean survival (± 1 S. E. M.) of *Calliphora vicina* adults provided with (PF) and without (PE) access to liver following 2 h exposure to progressively lower sub-zero temperatures (-4° to -12°C). Survival was assessed as successful adult eclosion at 11°C for larvae and spontaneous movement after 2 h recovery at 20°C for adults. n = 6 × 10 replicates per data point.

The greatest survival followed a pre-treatment of 3 h at 0°C. PE adults under the ecologically relevant thermoperiod cycle also significantly enhanced survival to DT exposure relative to DT (survival ranged between 53.3 ± 6.1% and 56.7 ± 4.2%; P<0.001; Tukey test). No RCH response was detected in PF adults following any pre-treatment (all Ps>0.05).

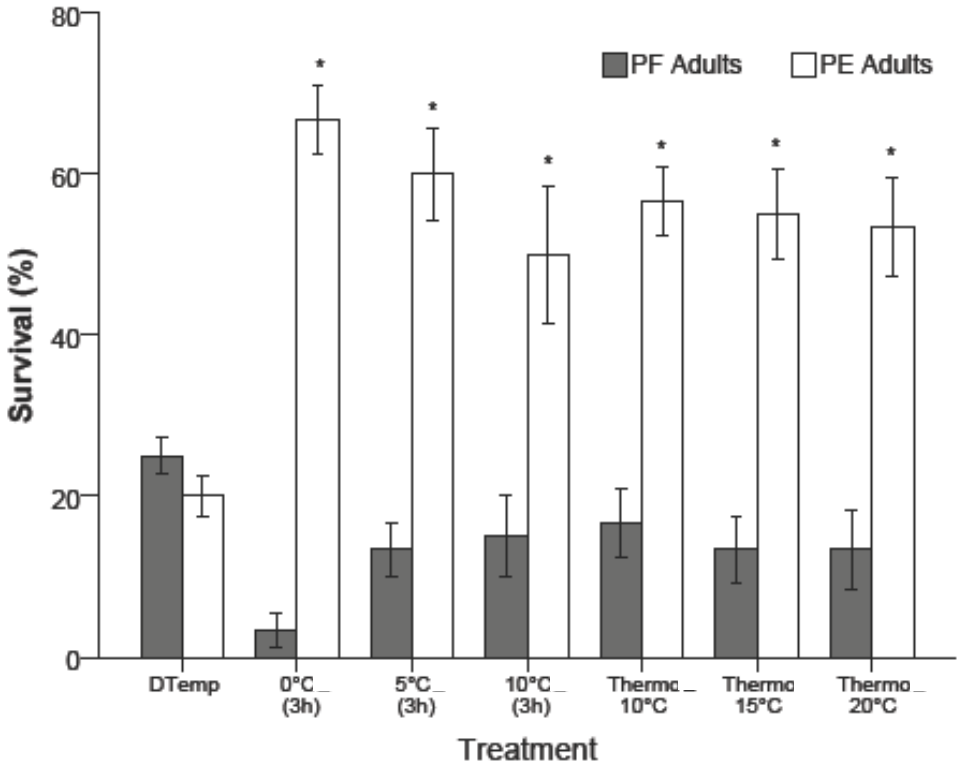

Figure 3.7 Mean survival (± 1 S. E. M.) as a measure of rapid Cold Hardening (RCH) ability of adult *Calliphora vicina* that have either been provided with (PF) or not provided with food (PE). RCH was determined following constant temperature pre-treatments or after a 6 d thermoperiod (thermo) at which adults were removed at 10°C, 15°C or 20°C. * significantly different from survival at the discriminating temperature (DT) (2 h at -10°C) for the same treatment at P<0 .05 . n = 6 × 10 replicates per data point.

3.4.7 CT_{min} and chill coma of adults

CT_{min} did not vary significantly in response to adult age or access to protein ($F_{3,56}$ = 13.08, P<0.05) (Table 3.2).

Adult chill coma temperatures varied significantly between different ages of PE adults ($F_{2,42}$ = 8.0, P<0.001), with d 0 adults entering chill coma at significantly higher temperatures than d 4 or d 6 adults. In addition, d 6 PF adults entered chill coma at significantly higher temperatures than d 6 PE adults.

Table 3.2 Mean temperature (± 1 S. E. M.) and range (in brackets) at which adult *Calliphora vicina* reach their minimum temperature for walking (CT_{min}) and entered chill coma. Adults ranged in age from zero to six days and had been provided with food or remained unfed, *n* provided in brackets. Means followed by 'a' are significantly different from the mean of 'PE adults on day zero' within the same column (Tukey's HSD, $P>0.05$)

Adult treatment	CT_{min}	Chill coma
PE adults (day zero)	-1.6 ± 0.1 (-3.7 to 2.7; *n*=14)	-5.9 ± 0.1 (-9.2 to -2.3; *n*=14)
PE adults (day four)	-1.4 ± 0.3 (-9.7 to 6.9; *n*=17)	-8.3 ± 0.1 [a] (-11.4 to -4.4; *n*=17)
PE adults (day six)	-3.2 ± 0.2 (-9.2 to 2.2; *n*=13)	-8.5 ± 0.2 [a] (-12.5 to 4.1; *n*=14)
PF adults (day six)	-0.3 ± 0.1 (-4.1 to 2.2; *n*=16)	-4.9 ± 0.1 (-8.9 to -0.8; *n*=16)

3.4.8 Adult SCPs

As with CT_{min}, the supercooling capacity did not vary significantly in response to age or feeding ($F_{3,114} = 7.40$, $P = 0.34$) (Table 3.3).

Table 3.3 Mean (± 1 S.E.M) and range (in brackets) supercooling points (SCP) for adult *Calliphora vicina*. Adults ranged in age from zero to six days and had been provided with food or remained unfed, *n* provided in brackets.

Adult treatment	SCP (°C)
PE adults (day zero)	-10.1 ± 0.6 (-5.4 to -16.4; *n*=30)
PE adults (day four)	-11.1 ± 0.6 (-5.4 to -16.5; *n*=24)
PE adults (day six)	-11.0 ± 0.6 (-4.4 to -17.7; *n*=32)
PF adults (day six)	-10.3 ± 0.5 (-6.3 to -15.7; *n*=32)

3.5 Discussion

Diapausing *C. vicina* larvae were intrinsically more cold tolerant than non-diapause larvae to direct cold exposure (DTs of 2h at -11°C and -10°C, respectively) (Figure 3.2). This agrees with previous studies (e.g. Saunders and Hayward, 1998), and builds on a body of evidence

in other insects linking enhanced cold tolerance with entry into diapause (Adedokun and Denlinger, 1985; Sobek-Swant et al., 2011), which is known to initiate multiple cold tolerance mechanisms (Denlinger, 2002; Hayward et al., 2005). Adults were less cold tolerant than larvae, which has also been identified in the kelp fly, *Paractora dreuxi* (Terblanche et al., 2007a) and the midge, *Belgica antarctica* (Lee et al., 2006) (Figure 3.2 and Figure 3.6). Evidence from *B. antarctica*, and other insects, suggests that while adults can relocate to more favourable micro-climates, larvae experience selective pressure to develop physiological adaptations *in-situ* to tolerate exposure to environmental stress (Rinehart et al., 2000; Hayward et al., 2007).

Both larvae and adults of the blow fly, *C. vicina*, clearly have the capacity to acquire protection against acute cold stress through RCH (Figure 3.3, Figure 3.4 and Figure 3.7). However, a stronger RCH response was exhibited by non-diapause larvae (Figure 3.3 and Figure 3.4), which may be because it is the non-diapause life stages that are typically exposed to more variable environments during late autumn or early spring, while diapause larvae typically overwinter in chronically cold but relatively stable subterranean microhabitats. A reduced RCH response has also been identified in overwintering life stages of the kelp fly, *P. dreuxi* (Terblanche et al., 2007a).

For both non-diapause and diapause larvae, survival at the DT increased following constant temperature pre-treatment at lower sub-zero temperatures, however, a pre-treatment of 1 h at 0°C followed by 1 h at -5°C failed to induce a RCH response (Figure 3.4). It is unclear why this pre-treatment failed to induce a response, especially as previous studies suggest that RCH is an ongoing process that starts to be initiated at temperatures well above 0°C and following pre-treatment times of less than 30 min (Denlinger and Lee, 2010). Nevertheless, constant temperature pre-treatments have been criticised as not representing conditions experienced *in-situ* (Bale, 2002). For this reason, the RCH response was also investigated using ecologically relevant cooling rates (Figure 3.4). Gradual cooling, as shown in Figure 3.4, has been associated with a stronger RCH response in species such as *E. murphyi*

(Everatt et al., 2012) and *D. melanogaster* (Kelty and Lee, 1999). It is believed that gradual cooling allows additional time for insects to reset thermal thresholds by implementing physiological adaptations, such as blocking of cold-induced apoptosis (Yi and Lee, 2011).

Increased DT survival is often associated with slower cooling rates (Kelty and Lee, 1999; Powell and Bale, 2004, 2006), but, for both non-diapause and diapause larvae, 0.5°C min^{-1} was the optimal cooling rate, with a weaker RCH response following slower (0.1°C min^{-1}) or faster (1°C min^{-1}) cooling (Figure 3.4). For non-diapause larvae, individuals cooled at 0.1°C min^{-1} to 0°C (followed by DT transfer) exhibited a stronger RCH response than individuals cooled at the same rate directly to the DT (76% and 50% survival, respectively). These findings suggest that it is time at low temperature rather than cooling rate which is most important in inducing the RCH response. Extended time at low temperatures, associated with slower cooling rates, increases the likelihood of encountering cold induced mortality, while cooling at a faster rate does not provide sufficient time for physiological adjustments to be fully implemented. Instead, intermediate time at low temperatures induces the strongest RCH response. Cooling at 0.5°C min^{-1} was also the optimal temperature for lowering the SCP of diapause larvae (-18.9°C) (Table 3.1) and lowered the lethal temperature of non-diapause larvae survival to -16°C (Figure 3.5). These findings suggest a close association between gradual cooling, RCH, supercooling capacity and enhanced low temperature tolerance.

For adults, the RCH ability was dependent on food intake (Figure 3.7). PF adults failed to exhibit a RCH response following any pre-treatment, including a 6 d thermoperiod. In contrast, PE adults displayed a strong cold hardening response following all temperature treatments. The strongest response followed 3 h at 0°C, with a 67% increase in survival compared to DT exposure. RCH in adult life stages is species-specific, with a response detected in *Euseius finlandicus* (Broufas and Koveos, 2001) and a number of Collembola species (Denlinger and Lee, 2010) but not in *P. dreuxi* (Terblanche et al., 2007a), or *B. antarctica* (Lee et al., 1996). Interestingly, PF adults also lost coordinated movement at higher temperatures than PE adults (Table 3.2). The reasons for this are not clear, but

feeding has been found to impair chill coma recovery in the migratory locust, *Locusta migratoria,* due to delayed K^+ and Na^+ regulation in the muscle and hemolymph, respectively (Andersen et al., 2013).

To the best of my knowledge, this is only the second study to find an effect of feeding on activity thresholds. The effect of feeding is unlikely to be a taxon-wide response with no consistent effect of adult feeding on cold tolerance detected in the butterfly, *Bicyclus anynana* (Franke et al., 2012). The relationship between adult feeding, activity thresholds and RCH suggests that RCH is part of a broader cooling response mechanism. For example, RCH in *Sitobion avenae* is associated with lowering of the CT_{min} (Powell and Bale, 2006), and in *D. melanogaster*, with enhanced courting behaviour and increased reproduction (Shreve, 2004). Early RCH studies were considered be a phenomenon restricted to laboratory experiments, but it is more likely to be an important ecophysiological adaptation that allows insects to 'reset' lower limits of survival over short time periods and enhance fitness over longer time scales (such as increased fecundity in subsequent life stages) (Sinclair and Chown, 2006; Kelty, 2007; Denlinger and Lee, 2010).

The role of RCH as a cold tolerance mechanism could become increasingly important as climate change continues. A loss of synchrony between temperature and photoperiod cues (Mousseau and Dingle, 1991; Mousseau and Fox, 1998) is expected to increase the proportion of insect populations aborting diapause, leaving life stages outside of diapause exposed to chronic cold (Bale and Hayward, 2010). Adult *C. vicina* certainly seem likely to experience winter mortality, given a DT temp of -7°C (Figure. 3.4) and an inability to move and thus feed at temperatures below -0.3°C (Table 3.2). Mean air temperatures from the 1st September 2010 until 31st January 2011 in Birmingham, UK (52.4°N) (BrumMet, 2013) fell below 0°C on 41 separate days. Over this period, there were four occasions when temperatures did not exceed 0°C for two or more consecutive days and the lowest temperatures recorded in 2009 and 2010 were -9.4°C and -12.45°C, respectively. Based on results presented in this chapter, adults that had previously accessed food would experience

high mortality at these low temperatures. The scarcity of food over the winter period suggests that adults exposed to cold may have stronger cold tolerance mechanisms, but it is yet to be determined how long adults can survive at low temperatures without feeding. However, there is some evidence that adults are able to acquire cold resistance through a reproductive diapause, although more research is needed to determine how this will affect winter survival (Vinogradova, 2009; Johnson, 2013). The RCH response exhibited by PE adults following a six day thermoperiod further substantiates the belief that RCH is an important cold-survival mechanism utilised on a regular basis by insects exposed to cold exposure on a diurnal timescale (Kelty and Lee, 1999).

3.6 Conclusions

There is growing evidence that diapause is being averted in a number of species residing within the temperate zone in response to continued global warming. The unpredictable nature of climate change means that increased temperatures, resulting in diapause aversion, can be followed by sudden periods of acute cold. The evidence presented here suggests that survival, following diapause aversion, during sudden periods of cold can be enhanced through the presence of a RCH response. Given the widespread occurrence of diapause and the presence of RCH in a substantial number of insect species investigated to date, it is likely that winter survival is already being enhanced through RCH within nature. However, while RCH response provides protection to periods of acute cold exposure, active life stages can still experience mortality at temperatures above those known to cause direct mortality. For example, at temperatures below the CT_{min}, adults are unable to relocate to more suitable environments or escape predation. For adults remaining active over the winter months, lower activity thresholds will be very important in determining the ecological limits of survival. These ecological limits are investigated in more detail in the subsequent chapter.

CHAPTER 4

THERMAL ACTIVITY THRESHOLDS OF THE BLOW FLY, *CALLIPHORA VICINA*, ARE INFLUENCED BY BOTH ACCLIMATION AND PHOTOPERIOD

4.1 Abstract

Insect responses to climate change will be determined, in part, by the proximity of temperatures experienced to the thermal limits of survival. For species with a wide geographic range, such as the blow fly, *Calliphora vicina*, this could include encountering both upper (CT_{max} and heat coma) and lower thermal thresholds (CT_{min} and chill coma). Within the temperate zone climate change is disrupting the timing of winter dormancies (diapause), leaving active life stages exposed to acute and chronic cold. In tropical locations it is increasingly likely that temperatures will exceed upper thermal thresholds. This chapter investigated the effect of adult photoperiod and acclimation temperature on the upper and lower activity thresholds of d 6 adult *C. vicina*.

Adult acclimation had a significant effect on lower activity thresholds. CT_{min} and chill coma for adults at 10°C were significantly lower than for adults at 20°C. There was no plasticity in upper thresholds, with a CT_{max} of 40-41°C and heat coma of 43°C, for all treatments. A shorter photoperiod was associated with lowering of activity thresholds, with CT_{min} and chill coma decreasing from 5.7 ± 1.1°C and -4.4 ± 0.8°C (under constant light) to 2.1 ± 0.7°C and -6.5 ± 0.9°C (under LD 12:12 h), respectively.

These findings indicate that both temperature and photoperiod play a role in setting activity thresholds. The effect of photoperiod in this regard has only been identified in one other study, however, underlying mechanisms require further investigation. The limited plasticity in the upper thermal thresholds, following acclimation, is in agreement with many other insect species.

4.2 Introduction

It is predicted that changes in temperature, in response to climate change, will exert a greater effect on insect fitness than changes in precipitation, CO_2 concentration or UVB

exposure (Bale et al., 2002). How insects and other ectotherms react to changes in temperature will be determined not just by the proximity of temperatures experienced to the thermal limits of survival (Chown and Gaston, 1999; Macmillan and Sinclair, 2011; Hoffmann et al., 2013), but also by how much they exceed activity thresholds, thereby limiting the ability to forage, mate and escape predation (Hazell and Bale, 2011; Alford et al., 2012; Everatt et al., 2013). Chapters 2 and 3 have focused on the absolute lower temperature limits of survival in adult and larval life stages. This chapter intends to expand upon the ecological limits of survival for the adult life stage, as introduced in Chapter 3 (section 3.4.7) and investigate the effects of temperature and acclimation on setting thermal thresholds.

Depending on the location, and season, climate warming may result in positive effects, such as the alleviation of low temperature stress during winter months at temperate latitudes (Bale and Hayward, 2010) or negative effects, such as upper thermal thresholds being exceeded during the summer months at tropical latitudes (Hoffmann et al., 2013). At temperate latitudes, the autumn and early winter is a particularly important period because many insects are still active, but are presented with potentially rapid diurnal fluctuations in temperature. This situation could be worsened as global warming disrupts the timing of winter dormancy (diapause) (Bale and Hayward, 2010) and temperature fluctuations become more frequent (Easterling et al., 2000; IPCC, 2013).

The survival of an active, feeding, life stage is dependent on temperatures remaining within a specific thermal range (see Chapter 1, section 1.2) (Macdonald et al., 2004; Alford et al., 2012). At temperatures either side of this thermal range, individuals are unable to locate food, disperse to more favourable locations, avoid predators or reproduce (Mellanby, 1939; Hazell et al., 2008). While short-term exposure to temperatures outside of the thermal range may not be disadvantageous to survival, long term exposure will eventually lead to mortality. For the majority of insect species, these thresholds are of particular importance in setting the limits of survival for the adult life stage. The adult life stage generally encounters a wider range of temperatures than larval life stages, which instead shelter in thermally stable

locations. The most commonly measured traits are the critical thermal minima (CT_{min}) and chill coma for activity at lower temperatures and critical thermal maxima (CT_{max}) and heat coma for activity at higher temperatures (Hughes et al., 2010; Hazell and Bale, 2011; Coombs and Bale, 2013).

As insects are cooled they pass through a well conserved set of physiological responses. At first, activity becomes impeded and walking speed decreases until the ability to maintain coordinated movement is lost, this point is called the CT_{min} (Cowles and Bogert, 1944). Beyond this point there is continued appendage movement, until the individual reaches a point of chill coma, signified by a final appendage twitch (Mellanby, 1939), although see Hazell and Bale (2011) for a review of different interpretations of the chill coma point. Chill coma is a reversible state with an increase in temperature resulting in regained abilities to move, including walking. If temperatures continue to decrease the individual will die.

As an insect is heated it passes through similar ecological thresholds to those experienced during cooling. At first coordinated movement is lost; this point is the CT_{max}. Beyond this point there is uncoordinated movement until the point of heat coma, signified by a final appendage twitch. Heat coma is usually synonymous with the upper limit of survival and is a non-reversible state.

Thermal thresholds often display a degree of phenotypic plasticity. Acclimation to sub-lethal temperatures (from hours to weeks) often extends these thresholds (Hoffmann et al., 2005; Rako and Hoffmann, 2006; Alford et al., 2012). There is typically a greater degree of plasticity in lower activity thresholds. Upper thermal thresholds are often already approaching the limits of metabolic activity and therefore the limits of survival (Hoffmann et al., 2013). Despite the frequent use of activity thresholds in temperature tolerance studies, the mechanisms underpinning these responses remain poorly understood. Based on current understanding, it is believed that chill coma is due to a failure to sustain ionic homeostasis at low temperatures, which leads to a loss of nerve and muscle sensitivities and a resultant inability to maintain locomotion (Macmillan and Sinclair, 2011). Upper temperature thresholds

are believed to be constrained by oxygen availability and a failure of macromolecules to maintain structure at high temperatures (Feder and Hofmann, 1999).

In addition to the effects of temperature on setting thermal activity thresholds, photoperiod may also be of importance (Vesala et al., 2012). This is because photoperiod plays a fundamental role in programming clock mechanisms (Tauber et al., 2007), which in turn control diurnal activity rhythms. In addition, changes in photoperiod underpin seasonal responses, such as diapause (Koštál, 2006). For *Calliphora vicina*, the adult life stage is believed to possess the ability to enter a form of reproductive diapause (Vinogradova and Zinovjeva, 1972), which raises the possibility that adult cold tolerance could be enhanced under short-day photoperiods. This has never been investigated. To the best of my knowledge, the effect of photoperiod on setting activity thresholds has only been identified in one previous study, in which a decreased day length experienced by adult *Drosophila montana* induced a cold acclimation response (Vesala et al., 2012).

Against this background, this chapter examined the upper (CT_{max} and heat coma) and lower (CT_{min} and chill coma) activity threshold of adult *C. vicina* and identified the effect of acclimation and photoperiod on setting these thresholds.

4.3 Materials and methods

4.3.1 Experimental cultures

Experimental cultures used in this chapter were established from the stock culture outlined in Chapter 2, section 2.3.1. *C. vicina* were originally sourced from the University of Birmingham campus, Birmingham, UK (52.4°N, 1.9°W) in 2009 and lab cultures regularly replenished with wild-caught individuals.

4.3.2 Adult acclimation

Experimental cultures were established using approximately 300 newly eclosed mixed sex adults in 30 cm^3 cages. Cultures were held in a programmable incubator under a short-day photoperiod (LD 12:12 h) at either 10°C, 15°C or 20°C. Sugar and water were provided *ad libitum* and adults used for experimental use on d 6.

4.3.3 Adult photoperiod

Experimental cultures were established using approximately 300 newly eclosed mixed sex adults under a temperatures of 15°C. Cultures were held under a photoperiod of constant light (LD 24:0 h), a long-day photoperiod (LD 18:6 h), and a short-day photoperiod (LD 12:12 h) or constant darkness (LD 0:24 h).

4.3.4 Thermal activity thresholds

Adult activity thresholds were determined using the temperature-controlled system developed by Hazell et al. (2008). For a detailed methodology refer to Chapter 3, section 3.3.6. Briefly, two adults were placed in a central arena (40 mm in diameter × 7.5 mm in depth) that allowed passage of temperature-controlled fluids from an attached alcohol bath (Haake Phoenix 11 P2, Thermo Electron Corp., Karlsruhe, Germany) which in turn altered the temperature in the adult arena.

To identify lower activity thresholds, adults were cooled from their acclimation temperature to 10°C at 0.5°C min^{-1} and then to -20°C at a rate of 0.2°C min^{-1}. Preliminary experimentation found this cooling rate to induce chill coma in 100% of individuals. Video recording software and constant temperature measurements were used to identify the temperature at which individuals entered CT_{min} (identifiable through a loss of coordinated movement) and chill coma (final appendage movement) (Cowles and Bogert, 1944). Upper activity thresholds were determined by warming adults from their acclimation temperature to 20°C at a rate of 0.5°C min^{-1} and then to 50°C at a rate of 0.2°C min^{-1}. Preliminary experimentation found this

rate to induce heat coma in 100% of individuals. CT_{max} and heat coma were identified during video playback. A minimum of eight replicates were used for each treatment.

4.3.5 Statistical analysis

Upper and lower thermal activity thresholds had to be calibrated against direct temperature recordings from immobile adults held within the cooling arena. Data were then analysed using a GLM analysis in SPSS (v. 20.0, IBM, New York, USA) and significant differences between groups were identified using the conservative Bonferroni post-hoc test with an alpha threshold of $P<0.05$.

4.4 Results

4.4.1 CT_{min} and chill coma

4.4.1.1 The effect of acclimation

Significant differences were detected in the CT_{min} and chill coma of d 6 adults acclimated to either 10°C, 15°C or 20°C ($F_{2,22}$ = 13.17, $P<0.001$ and $F_{2,24}$ = 5.54; $P<0.001$, respectively) ($F_{2,22}$ = 13.17, $P<0.001$ and $F_{2,24}$ = 5.54; $P<0.001$, respectively) (Figure 4.1).

Adults acclimated to 10°C had the lowest mean CT_{min} temperature, and post-hoc analysis identified this to be significantly lower than CT_{min} exhibited by adults acclimated to 15°C ($P<0.01$) or 20°C ($P<0.001$).

Similarly, adults acclimated to 10°C reached chill coma at the lowest temperature. Post-hoc analysis identified this to be significantly different from 20°C ($P<0.05$) but not 15°C (P=0.74) acclimated adults.

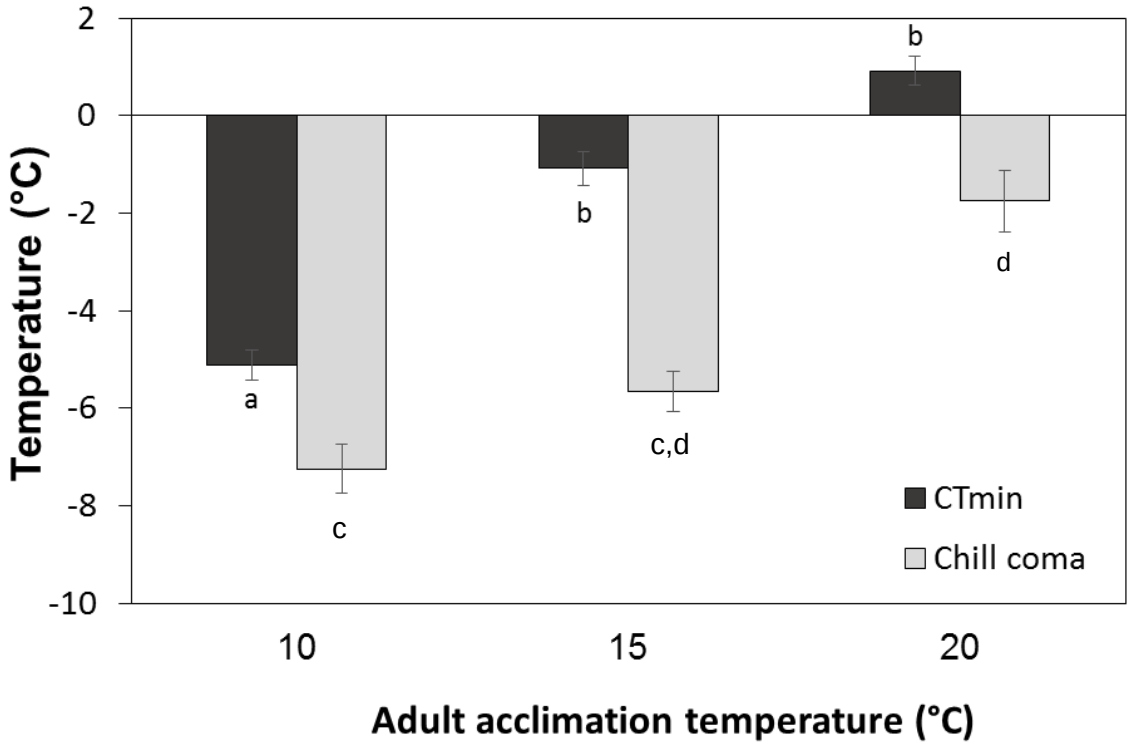

Figure 4.1 The mean temperature (± 1 S. E. M.) at which 6 day-old adult *C. vicina* reached their minimum temperature for walking (CT_{min}) and entered chill coma. Adults were acclimated to either 10°C (*n* = 7), 15°C (*n* = 7 for CT_{min} and 9 for chill coma) or 20°C (*n* = 8). Means associated with different letters are significantly different from each other.

4.4.1.2 The effect of photoperiod

The CT_{min} of d 6 adults varied significantly between photoperiod treatments ($F_{3,55}$ = 4.24; *P*<0.001), with a general decrease in CT_{min} as daylight hours were shortened. Post-hoc analysis identified a significant difference between adults under constant light and those cultured under a short-day photoperiod (LD 12:12 h) (*P*<0.05), (Figure 4.2).

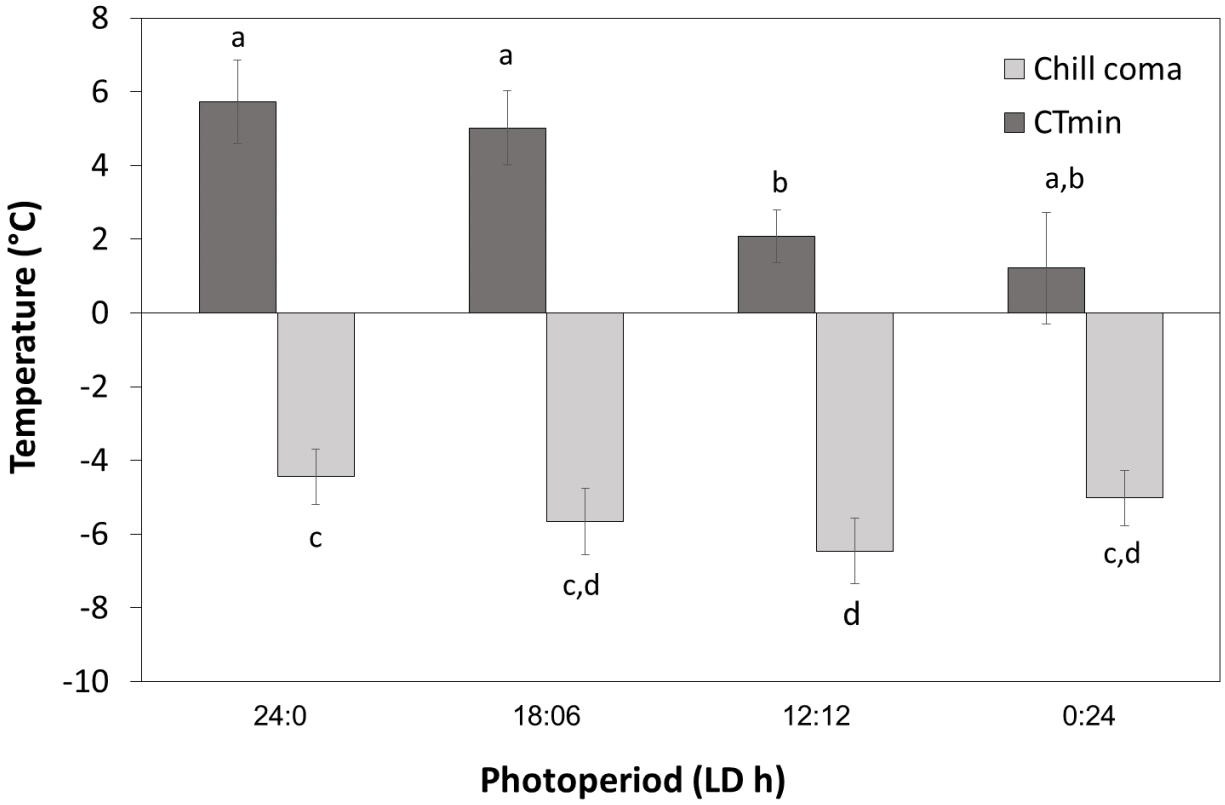

Figure 4.2 The mean temperature (± 1 S. E. M.) at which 6 day-old adult *C. vicina* reached their minimum temperature for walking (CT_{min}) and entered chill coma. Adults were acclimated to 15°C under either constant light (LD 24:00 h) (*n* = 13), a long-day photoperiod (LD 18:06 h) (*n* = 15), a short-day photoperiod (LD 12:12 h) (*n* = 20) or total darkness (LD 00:24 h) (*n* = 7). Means associated with different letters are significantly different from each other.

Photoperiod had a significant effect on the temperature that adults entered chill coma ($F_{3,55}$ = 4.24, *P*<0.01). Post-hoc analysis identified this difference to be significant between adults under complete darkness (LD 24:0 h) and adults under a short-day photoperiod (LD 12:12 h) (*P*<0.05).

4.4.2 CT_{max} and heat coma

The upper temperature thresholds of CT_{max} and heat coma were not significantly different between 6 d old adults acclimated to 10°C (CT_{max} $F_{1,15}$ = 2.6, *P*=0.13; heat coma $F_{1,15}$ = 0.02, *P*=0.90) or 20°C, see Figure 4.3.

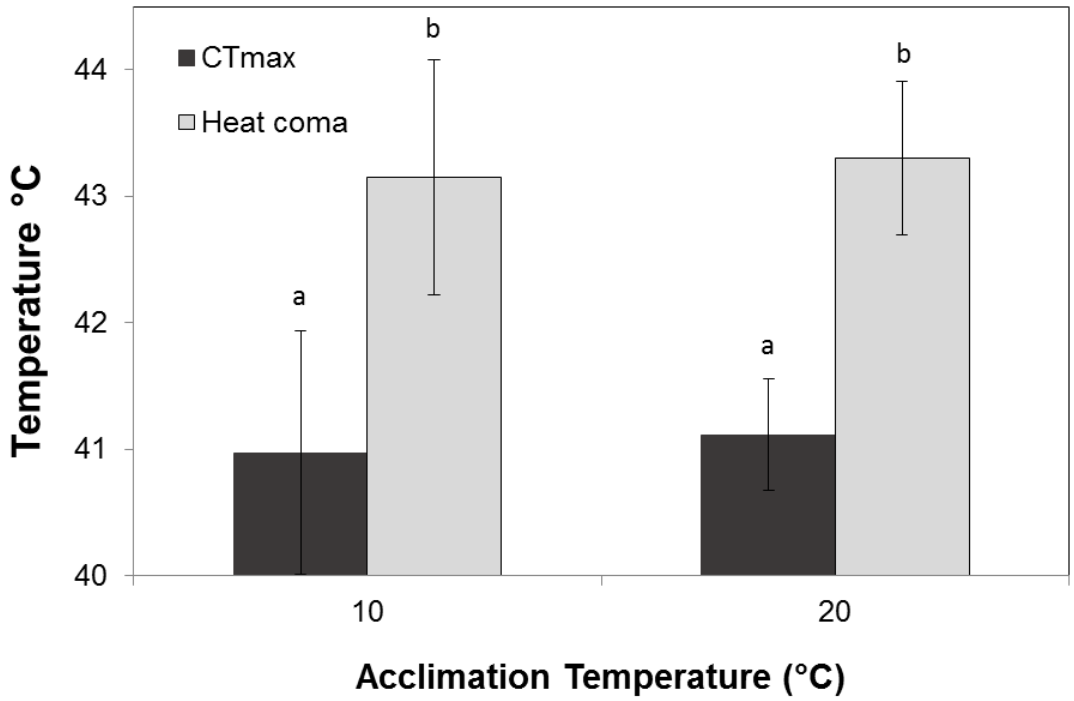

Figure 4.3 The mean temperature (± 1 S. E. M.) at which 6 day-old adult C. vicina reached their maximum temperature for walking (CT_{max}) and entered heat coma. Adults were acclimated to either 10°C or 20°C Means associated with different letters are significantly different from each other. *n* = 8 for each CT_{max} and 7 for each heat coma group.

4.5 Discussion

Insect responses to climate change will be determined by the proximity of temperatures experienced to the thermal limits of survival (Chown and Gaston, 1999; Macmillan and Sinclair, 2011; Hoffmann et al., 2013) and the phenotypic plasticity of these thresholds. The findings presented in this chapter illustrate that there is considerable plasticity in the lower activity threshold of adult *C. vicina* (Figure 4.1). Adults acclimated to 10°C entered CT_{min} and chill coma at -5.1°C and -7.2°C, respectively. These values were 6°C and 5.4°C lower than for adults acclimated to 20°C. Acclimation has been shown to have a similar effect on lowering the activity thresholds of numerous insect groups, including *Drosophila* spp. (Gilbert et al., 2001) and aphid (Hazell et al., 2010; Alford et al., 2012) species. There is considerable variation in lower activity thresholds of insects, ranging from around -16°C (CT_{min}) in the cold

tolerant midge, *Diamesa meigen* (Kohshima, 1984) to above 20°C for tropical insects, such as the tsetse fly, *Glossina pallidipes* (Terblanche et al., 2007b).

The acclimation response presented in this chapter would prove beneficial if non-lethal cold temperatures are indicative of lower temperatures to come, as suggested by the beneficial acclimation hypothesis of Huey et al. (1999). For instance, adults active over winter months would benefit from an ability to maintain locomotion at lower temperatures than summer acclimated adults, as they are more likely to encounter sudden periods of low temperature. Equally, this adaptation would prove beneficial over the geographic range of *C. vicina*. Individuals at higher latitudes (where average temperatures are lowest) would be better equipped for periods of low temperature than individuals at lower latitudes. However, to confirm that adults at higher latitudes are able to maintain locomotion at lower temperatures than individuals at lower latitudes, would require a latitudinal comparison between different geographic strains. Insects often express a latitudinal variation in thermal thresholds. Populations of *Drosophila melanogaster* from higher latitudes (e.g. along the east coast of Australia) show increased resistance to cold shock and maintain activity at lower temperatures than populations at lower latitudes (Hoffmann et al., 2002). In general, insect species exhibit a pole-ward increase in cold hardiness to compensate for the increased frequency of low temperature events (Hoffmann et al., 2002). This response provided positive adaptive traits to local environmental conditions

Acclimation had no effect on the upper thermal thresholds, with a CT_{max} and heat coma of approximately 41°C and 43°C, respectively, regardless of acclimation temperature (Figure 4.3). These findings are in accordance with numerous other studies which have identified greater plasticity in lower activity thresholds (Chown, 2001; Hughes et al., 2010; Alford et al., 2012; Hoffmann et al., 2013). Examples include, the aphid, *Myzus persicae*, which displays considerable plasticity in lower activity thresholds but only a very limited plasticity in its upper thresholds (Alford et al., 2012). For *M. persicae*, acclimation to 10°C reduced the lower

activity threshold from 8.8°C to 2.5°C, while acclimation to higher temperatures (25°C) only extended the upper activity threshold from 41.4°C to 42.3°C.

Upper thresholds typically range between 40°C and 50°C and plasticity in these limits rarely exceed 1°C following acclimation (Chown and Nicolson, 2004; Alford et al., 2012; Hoffmann et al., 2013). It is believed that at temperatures above 50°C, intracellular oxygen becomes limiting and macromolecular structures become destabilised (Feder and Hofmann, 1999; Hoffmann et al., 2013). Within the UK it is unlikely that temperatures will exceed the upper thresholds for *C. vicina*. However, at lower latitudes, temperatures could exceed upper activity thresholds as climate change continues.

Photoperiod, like temperature, had a modifying effect on the temperature that adults reached CT_{min} and chill coma (Figure 4.2). CT_{min} decreased from 5.7°C in d 6 adults under complete light to 1.2°C in adults under complete darkness. Chill coma decreased from -4.4°C under complete light to -6.5°C under a short-day photoperiod (LD 12:12 h). A similar relationship between photoperiod and activity has been identified in *D. montana*, in which short-day conditions experienced by the adult life stage were associated with an increase in the cold tolerance phenotype, such as chill coma recovery time (Vesala et al., 2012). This was believed to be an adaptive trait, induced under photoperiods naturally experienced *in-situ* over the winter months to enhance activity under seasonally low temperatures (Vesala et al., 2012).

Photoperiod underpins the regulation of numerous traits in insects, such as growth rate (Saunders, 1972) and the programming of diapause (Tauber and Kyriacou, 2001). The programming of diapause involves an ability to detect a specific day length, a photoperiod clock, that can distinguish between day and night length, a counter mechanism, and a neurohormonal output that programmes diapause (Koštál, 2011). For the majority of temperate insects, the programming of diapause is associated with an enhanced ability to tolerate low temperature exposure (see Chapters 2 and 3). It is therefore possible that the increased cold tolerance, induced by a short-day photoperiod, is an indication of an adult

diapause. The presence of an adult diapause has been identified in *C. vicina* populations at high latitudes (Vinogradova, 2011). Also, a tendency for group aggregation at low temperatures indicates the presence of an adult diapause in the Birmingham strain (Johnson, 2013). However, without further investigation, it is not possible to determine whether the response identified here is part of an adult diapause phenotype.

4.6 Conclusions

The findings presented in this chapter could have important implications for *C. vicina* as climate change continues. The effect of acclimation to low temperatures in lowering the point of CT_{min} by 6°C suggests that winter-active adults, following diapause disruption, will be able to maintain coordinated movement at lower temperatures than summer-acclimated individuals. The ability to maintain movement over the winter months is further enhanced by a positive response to short-day length. It is unclear how day length influences lower activity thresholds, but, it could be the result of an adult diapause under the control of photoperiod.

Upper temperature thresholds (41-43°C) are unlikely to be exceeded on a regular basis within the UK, and even on occasions when these thresholds are surpassed, behavioural adaptations such as relocating to cooler habitats are likely to be employed. However, the limited plasticity of upper thermal thresholds highlights the vulnerability of insects to periods of high temperature stress.

The following chapter uses *in-situ* temperature measurements to calculate the duration for which temperatures were below the chill coma and CT_{min} data over the winter months between 2009 and 2013. This information is then used, in combination with a phenological study, to determine what environmental factors are most likely to disrupt the timing of diapause as climate change continues.

CHAPTER 5

CLIMATE-DRIVEN CHANGES IN WINTER ACTIVITY OF THE BLOW FLY, *CALLIPHORA VICINA*: USING LABORATORY PHYSIOLOGICAL INDICES TO PREDICT PHENOLOGY AND SURVIVAL

5.1 Abstract

In the blow fly, *Calliphora vicina*, the induction, maintenance and termination of larval diapause are influenced by temperatures experienced by both diapause and non-diapause life stages. This makes it particularly susceptible to the effects of climate change. Based on this understanding, the *in-situ* seasonal phenology of *C. vicina* (timing and duration of diapause) was investigated in response to air and soil temperature fluctuations. Over all years of the study (2009 to 2013 inclusive), the induction of diapause was delayed beyond maternal detection of the specific photoperiod used to induce diapause in early-September.

A positive relationship existed between soil temperature (August to October experienced by L3 larvae) and adult abundance in November. Diapause was most delayed in 2010 (adults observed in December), a year when soil temperatures most frequently (i.e. 76 d) exceeded 15°C. The relationship between larval temperature and diapause aversion was confirmed under laboratory conditions, with larvae at 15°C completing diapause in under 8 d.

The factors influencing the termination of diapause appear to complex and influenced by multiple environmental factors. Under laboratory conditions, 50% of larvae completed diapause in under 64 d at 5°C. *In-situ* average soil temperatures varied between 3.2°C (2010) and 6.8°C (2011) and adults were absent for two months over each year, suggesting that spring emergence could be a result of the duration of diapause under *in-situ* soil temperatures. Spring emergence could therefore be influenced by the timing of diapause induction.

Finally, the absence of adults during January, when temperatures below chill coma and CT_{min} were most frequent, appeared to be a pre-emptive response, reinforcing the belief that diapause is an anticipatory response to low temperatures.

5.2 Introduction

Indications of human induced climate change are now unequivocal (IPCC, 2013), as is evidence that climate change is having significant effects on the Earth's biota (Parmesan, 2006). In particular, higher temperatures have been identified as the leading cause of species' loss (Barnosky et al., 2011), range expansions (Musolin et al., 2010; Otaki et al., 2010; Walther, 2010) and alterations in the timing of yearly biological events (phenology) (Parmesan and Yohe, 2003; Visser and Both, 2005; Parmesan, 2006). Leaf emergence on trees in the Mediterranean has advanced by 16 days over 50 years (Penuelas et al., 2002); average egg laying onset in UK birds has advanced by nine days over 25 years (Crick et al., 1997), and breeding in Canadian red squirrels has advanced by 18 days over 10 years (Réale et al., 2003). Similar advancements in yearly events have been documented around the globe, with a general advancement in the timing of spring events in response to higher temperatures (see Penuelas and Filella [2001] for a short review). A meta-analysis of over 1500 species concluded that approximately half (57%) of species have exhibited shifts in phenology or distribution, in accordance with changes in climate (Parmesan and Yohe, 2003). On average, spring events are advancing at 2.3 days per decade while pole-ward range boundaries are advancing at 6.1 km per decade (Parmesan and Yohe, 2003; Parmesan, 2006).

The close association between temperature and life history, for a range of taxa, makes phenological studies invaluable in documenting and predicting responses to climate warming (Roy and Sparks, 2000), as well as in understanding the interconnectivity between species and the environment (Forrest and Miller-Rushing, 2010). This is especially true for poikilothermic organisms, such as insects, whose development, phenology and survival are all closely tied to environmental temperature. Studies of insects are particularly important because these organisms play fundamental roles in key ecosystem services such as pollination and nutrient recycling (Losey and Vaughan, 2006). They also represent major

pests in agriculture (Zhang et al., 2007) and vectors of disease (Manachini et al., 2013; Oliveira et al., 2013; Shaw et al., 2013).

Models outlining the impact of climate change on insect phenology have typically been generated from large scale, and long running, datasets observing shifts in the spring emergence of 'charismatic' insects such as bees (Hymenoptera) (Potts et al., 2010; BWARS, 2013), butterflies (Lepidoptera) (Hassall et al., 2007; Butterfly Conservation, 2013), hoverflies (Diptera) (Graham-Taylor et al., 2009; Dipterists Forum, 2013) and dragonflies (Odonata) (Richter et al., 2008; British Dragongly Soceity, 2013). Understandably, such long-term records are not available for the changing distribution and seasonal activity of most insect species. Nevertheless, even small-scale studies of single species have provided an important insight into impacts of climate change. Range expansions in the legume and soybean pest, *Nezara viridula* (Musolin, 2007), the duration of summer dormancy in the solitary bees, *Osmia cornuta* and *O. lignaria* (Sgolastra et al., 2012) and the depletion of energy reserves in overwintering life stages of *O. lignaria* (Sgolastra et al., 2011) have all been identified in short-term studies. Small-scale studies can be particularly important in trying to determine how climate warming may alter insect overwintering. This consideration is lacking from most long-scale studies because winter dormancy or activity is rarely chronicled in studies that have been running over multiple years.

For the majority of temperate insects an important *in-situ* adaptation to seasonal change is diapause. An important aspect of diapause, in relation to climate change, is the modifying effect of temperature. This includes temperatures experienced by the sensitive stage, as well as subsequent developmental stages prior to and during diapause (Denlinger, 2002; Hahn and Denlinger, 2011). For a number of species, temperatures must remain development thresholds during diapause induction, otherwise diapause incidence will be reduced, and in some instances averted completely (Tauber et al., 1986; Vaz Nunes and Saunders, 1989; Tachibana and Numata, 2004b; Vinogradova, 2011). For *C. vicina* and *L. sericata*, adults

exposed to diapausing inducing photoperiods, but temperatures above 25°C, will not produce diapausing progeny (Tachibana and Numata, 2004a; Vinogradova, 2011; Johnson, 2013).

In *C. vicina*, there is no sensitivity to temperatures during the larval feeding stages (L1, L2 and L3-early) (Chernysh et al., 1995), which is thought to be because the feeding mass regularly experiences temperatures higher than those of the surrounding environment. This is due to heat generated by enzymes secreted by the larvae and digestive microorganisms on the food (Chernysh et al., 1995). However, larvae regain sensitivity to temperature during the wandering (3rd instar larval) stage. Exposure to temperatures above 15°C or 20°C, for *C. vicina* and *L. sericata*, respectively, result in diapause being averted (Vaz Nunes and Saunders, 1989; Tachibana and Numata, 2004a).

Temperatures experienced during the sensitive stage can also have an effect on diapause duration, and thus the timing of spring emergence (Bale and Hayward, 2010; Hahn and Denlinger, 2011). Adult *C. vicina* acclimated to 20°C under short-day conditions produce fewer diapausing offspring with a shorter diapause duration than adults acclimated to 15°C (McWatters and Saunders, 1998). A similar response is observed in *L. sericata* (Tachibana and Numata, 2004b). Temperature experienced by the diapause life stage itself also affects the duration of diapause. For example, pupal diapause in *Sarcophaga crassipalpis* is maintained for 118 days at 12°C compared to only 57 days at 28°C (Denlinger, 1972). The close association between temperature and diapause duration is a likely response to increased metabolism and the rapid depletion of energy and nutrient reserves as temperatures increase (Hahn and Denlinger, 2011). Sufficient energy reserves are not only required to sustain diapause, but also to instigate development once favourable conditions have returned in the spring (Bale and Hayward, 2010; Hahn and Denlinger, 2011). This is especially true for insects overwintering as post-fed larvae or pupae, for which the entire process of metamorphosis must ensue before there is an opportunity to feed again.

Under field conditions, diapause often ends in mid-winter (e.g. in early January for *Sarcophaga bullata*) (Denlinger, 1972). Once diapause has ended, many species enter a

period of post-diapause quiescence during which the cold tolerance mechanisms associated with diapause are still in place, diapause-associated genes continue to be expressed and metabolism continues to be kept at a minimum (Hayward et al., 2005). Post-diapause quiescence can be maintained for weeks to months, with development being resumed either spontaneously or following detection of environmental stimuli indicative of spring (Koštál, 2006).. For *C. vicina*, development is resumed spontaneously if low temperatures are sustained, or if environmental temperatures exceed the developmental threshold of 15°C (Vaz Nunes and Saunders, 1989). Once post-diapause quiescence has completed the cold tolerance associated with diapause is lost and continued development is resumed (Hayward et al., 2005).

For a number of species, elevated late-autumn and early-winter temperatures are already resulting in a loss of synchrony between the photoperiod and temperature cues used to induce diapause, resulting in diapause abortion (Bale and Hayward, 2010). The abortion of diapause results in resumed development, increasing the likelihood of non-diapause life stages encountering periods of winter cold, without cold tolerance mechanisms in place. Once diapause has been aborted, another generation must be completed, and diapause initiated later in the year to avoid non-diapause life stages being exposed to winter cold (Bale and Hayward, 2010). However, completion of an additional life cycle will not always be possible due to limited thermal budgets to complete another generation (Musolin, 2012). The winter survival of larval and adult life stages, outside of diapause, will be determined by different environmental factors. For non-feeding larval life stages survival will mainly depend on temperatures remaining above those capable of causing direct and indirect chilling injury (Denlinger and Lee, 2010). For adult life stages, winter survival is more complex, and survival is dependent not only on temperatures remaining above thresholds capable of causing direct mortality but also on temperatures remaining above minimum activity thresholds, thus enabling individuals to relocate to more favourable environments, locate food, avoid predation, or possibly even reproduce (Hughes et al., 2010; Macmillan and

Sinclair, 2011; Coombs and Bale, 2013; Everatt et al., 2013;). These thresholds were covered in detail in Chapter 4. Briefly, the two most commonly measured activity thresholds are the CT_{min} and chill coma (Hughes et al., 2010; Hazell and Bale, 2011; Coombs and Bale, 2013). CT_{min} is signified by the temperature at which coordinated movement is lost (Cowles and Bogert, 1944), while chill coma is represented by a final appendage twitch (Mellanby, 1939). Beyond the point of chill coma the insect is in complete paralysis (Hazell and Bale, 2011) although it is a reversible state and if temperatures increase then the ability to walk is regained. However, if temperatures continue to decrease the individual will eventually succumb to mortality.

Against this background, this chapter aims to:

1. determine the effect of adult and larval temperature on the incidence and duration of diapause in the blow fly, *C. vicina* in the laboratory,
2. document the seasonal phenology of adult *C. vicina* over four years, in combination with details of seasonal air and soil temperature fluctuations, to determine how the timing and duration of diapause under field conditions were influenced by environmental temperature,
3. combine the outputs of objectives 1 and 2, to determine if laboratory-based indices of physiology characterising diapause disruption by temperature allow accurate predictions of the phenology of *C. vicina* under field conditions.

Consequently, it was hypothesized that diapause would be delayed in years where late summer/autumn mean air temperatures exceeded 20°C or soil temperature exceeded 15°C.

Warmer autumn conditions (during induction) and/or winter temperatures (during diapause) are likely to reduce diapause duration (Chernysh et al., 1995; Johnson, 2013). It was therefore hypothesised that warmer winters would be followed by earlier spring emergence of adult flies (Figure 5.1).

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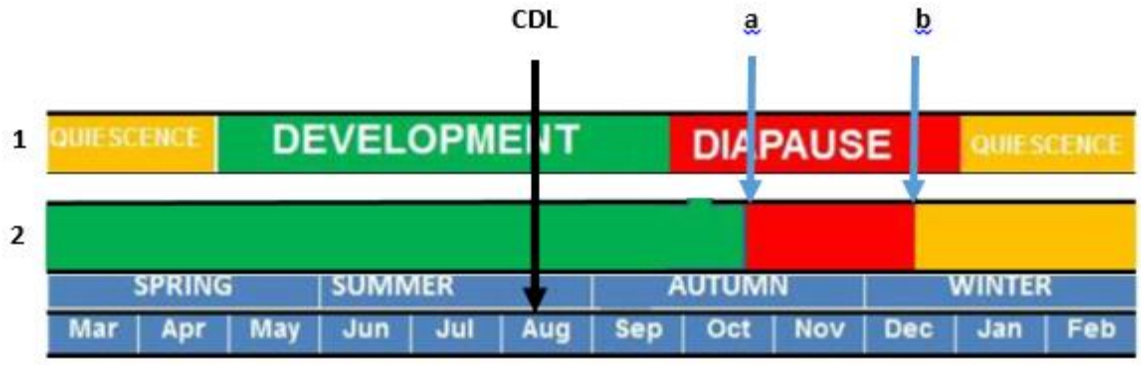


Figure 5.1 Graphical representation of the possible effect of climate change on diapause induction in *C. vicina*. Line **1** represents development through the growing season (green), diapause (red) and post-diapause quiescence (yellow) under present day conditions. The black arrow shows timing of the critical day length (CDL) (at 52°N) in late August. Line **2** represents the effect of elevated (soil and air) temperature on the induction of diapause, with elevated temperatures (blue line labelled '**a'**) delaying entry into diapause or causing complete aversion and (blue line labelled '**b'**) shortening the duration of diapause, resulting in the development being resumed earlier in the year.

The seasonal changes of *L. sericata*, *Musca domestica* (the house fly)*, Sarcophaga carnaria* (the common flesh fly), *Sylvicola fenestralis* (the window gnat) and *Dryomyza anilis* (a common carrion-feeding fly) were also recorded. The CDL of the Birmingham strain of *L. sericata* is not known, but it was hypothesised that diapause induction in this species would be less susceptible to disruption by elevated temperatures. This is due to a day-length sensitivity in larval stages of *L. sericata*, with exposure to short-day photoperiods reinforcing the cue to enter diapause (Tachibana and Numata, 2004b). While no studies have been performed on *S. carnaria*, *S. fenestralis* or *D. anilis* to determine if they have the capacity to enter a winter diapause, it is assumed here that they do enter diapause, in common with most temperate insects (Tauber et al., 1986; Denlinger, 1991), and that winter-active adults would again be a good indicator of diapause disruption.

The final objective of this chapter (4) was to determine if the activity thresholds for adult *C. vicina* identified in Chapter 4, could be used to predict periods of adult winter activity in

individuals where diapause has been averted. In this instance, it was hypothesized that the abundance of adults would decrease as the frequency (number of days) and duration (number of hours) of events below the chill coma and CT_{min} increased and adults were unable to maintain locomotion.

5.3 Materials and methods

5.3.1 Experimental cultures

The protocol for culturing adults and larvae followed that outlined in Chapter 2, section 2.3.2. Adults cultured under a long-day photoperiod (LD18:6 h) produced predominantly non-diapause larvae while adults under a short-day photoperiod (LD 12:12 h) produced predominantly diapause larvae. Adults were maintained from the point of eclosion at either 15°C or 20°C to produce non-diapause (abbreviated to ND15, ND20, respectively) and diapause (abbreviated to D15 and D20, respectively) L3 larvae.

5.3.2 The effect of temperature on diapause incidence in the laboratory

Diapause incidence was determined for all treatments (ND15, ND20, D15 and D20). Groups of 100 third-instars were transferred into a 5 cm × 5 cm plastic container containing a thin layer of sawdust, at the time of mass wandering (approximately 12 days post-eclosion). Larvae were held under complete darkness at 11°C until 30 d post-oviposition, which represents day zero (d 0) of diapause (Saunders, 1987). At this time, the number of larvae that had not developed to the pupal life stage was taken to signify the proportion of larvae in diapause. There were $n = 100 \times 4$ for each treatment.

5.3.3 The effect of temperature on diapause in the laboratory

Diapause duration was determined for D15 and D20 larvae only. On d 0 of diapause, groups of 10 third instars were placed into a Petri dish containing a thin layer of sawdust and

transferred to either 5°C, 10°C, 15°C, 20°C or 25°C (*n* = 10 × 9 for each treatment) under complete darkness. The number of larvae remaining within diapause (identified as those individuals which had not pupariated) was recorded daily, until all larvae had reached the pupal life stage. Cumulative pupariation was thus used to calculate the time taken for 10, 50 and 90% of each cohort to terminate diapause under the five treatment temperatures. This protocol was repeated twice to provide *n* = 18 × 10 for each treatment.

5.3.4 Climate data

Temperature data were recorded at 1 h intervals from the 1st October 2009 until the 30th April 2013 by the University of Birmingham Climate and Atmospheric Resources Group Weather Facility (BrumMet, 2013) (52.4°N, 1.9°W). A permanent weather station was used to measure air temperature (as would be experienced by adults) at a height of 20 cm, and soil temperature (as would be experienced by L3 larvae) at a depth of 10 cm. These data were then used to calculate the frequency (number of days) and duration (number of hours) that air temperature exceeded 25°C, 20°C and 15°C, and also the frequency and duration of temperatures below 0°C. For soil temperature, the frequency and duration of temperatures exceeding 15°C and 10°C and below 0°C were determined. This was determined for the temperatures experienced by adults during the initiation of diapause (August to October), during the larval maintenance of diapause (November and December) and during post-diapause quiescence (January to March).

5.3.5 C. vicina phenology

The abundance of adult *C. vicina* was recorded every month from October 2009 to April 2013, approximately 25 m from the location that climate data were collected. Flies were collected using an olfactory trap (Figure 2.1).

Each trap was constructed from two 1.5-L clear plastic drink bottles to produce a lower bait chamber and an upper collection chamber. The lower bait chamber contained 25 g of pig

liver and 15 ml of sodium hydroxide, to act as a chemical stimulus for adult carrion-feeding flies, and contained approximately 10 entry holes to allow passage into the chamber.

Flies enter the bait chamber, attracted to the liver and sodium hydroxide, and then naturally fly up towards the collection chamber through an inverted bottle lid. Flies then became trapped in the collection chamber and attached to a 10 cm × 5 cm glue board trap (Russell IPM, London, UK).

Seven identical traps were placed at 6 m intervals along a 42 m transect deciduous woodland on the University of Birmingham campus (52.4°N, 1.9°W) between the 10th to 15th day of each month. Traps were placed in the same position each month, at a height of 2 m, where they were left for a period of one week. After one week they were transferred directly to -20°C for a minimum of 48 h to kill the captured flies. The number of adult *C. vicina* in each trap was then recorded.

5.3.5.1 Adult activity in November (evidence of reduced diapause incidence)

Active adults (caught in traps) during November of each year were used as evidence that diapause had been averted in late August/September when field populations would have first experienced the CDL. Differences in the mean abundance of adult *C. vicina* in November between years were then correlated against specified temperature thresholds (see below) to identify which parameters best-explained differences in diapause incidence across years. The temperature thresholds correlated against adult abundance were:

- the average air temperature in the month directly preceding traps being placed in the field,
- the frequency that air temperature exceeded 20°C between 1st August and 30th October,
- the frequency that air temperatures (number of days) exceeded 20°C in September,

- the frequency that soil temperatures exceeding 15°C between 1st August and 30th October.

5.3.5.2 Adult activity in March (evidence of early diapause termination)

Variations in the mean abundance of adult *C. vicina* in March between years was correlated against temperature thresholds to identify which parameters best-explained adult abundance. The temperature thresholds correlated against adult abundance were:

- the average air temperature in the month directly preceding traps being placed in the field.
- the frequency that soil temperatures exceeded 15°C between November and December,
- the frequency that soil temperature exceeded 5°C from January to March, the frequency that soil temperatures fell below 0°C from November to March.

5.3.6 Frequency and duration of temperatures above and below CT_{min} and chill coma

The frequency and duration that temperatures fell below the CT_{min} and chill coma for adult *C. vicina* on the University of Birmingham campus were calculated using constant temperature data from October 2009 to April 2013. The CT_{min} and chill coma were determined in Chapter 4 using 6 d old adult *C. vicina* acclimated to 20°C under a short-day photoperiod (0.9°C and -1.7°C, respectively). The number of days over each month that temperatures fell below the CT_{min} and chill coma were used to produce the frequency data. The total number of hours, over each month, that average temperatures remained below the CT_{min} and chill coma were then used to determine the total duration of temperatures below the CT_{min} and chill coma.

5.3.7 Field trapping to monitor phenology of additional species

The abundances of adult *L. sericata*, *M. domestica*, *D. anilis*, *S. carnaria* and *S. fenestralis* were recorded at the same time as recording adult *C. vicina* following the same procedure as outlined in section 1.3.5. For all species, monthly abundance was recorded using olfactory traps from October 2009 to April 2013 inclusive.

5.3.8 Statistical analysis

Differences in diapause incidence across laboratory temperature treatments and differences in the abundance of active adults in March and November within the field studies were analysed using SPSS (v. 20.0, IBM, New York, USA). Diapause incidence were analysed using GLMs. Significant differences between data groups were identified using the Bonferroni *post-hoc* test with an alpha threshold of 0.05. Frequency data (number of days that soil temperature exceeded 15°C and number of days that soil temperatures were below 0°C) were analysed using GLMs and significant differences between groups were identified using the Bonferroni post-hoc test with an alpha threshold of $P<0.05$.

Differences in diapause duration between laboratory temperature treatments were analysed using the statistical package Minitab 15 (Minitab, Coventry, UK). A probit analysis based on Finney (1971), was used to determine the time taken for 10, 50 and 90% pupariation/diapause termination. Differences were identified between treatments through identification of non-overlapping upper and lower percentiles (± 95% fiducial limits) as in Hart et al. (2002).

5.4 Results

5.4.1 The effect of temperature on diapause incidence in the laboratory

Parental acclimation did not have a significant effect on the number of larvae entering diapause under a short-day photoperiod ($F_{2,6} = 3.32$, $P=0.118$), with a high incidence of

diapause for both D15 and D20 larvae. Fewer larvae entered diapause when parents were cultured under a long-day photoperiod and there was a significant difference between ND15 and ND20 larvae ($F_{2,6} = 10.79$, $P<0.05$) (Figure 5.2).

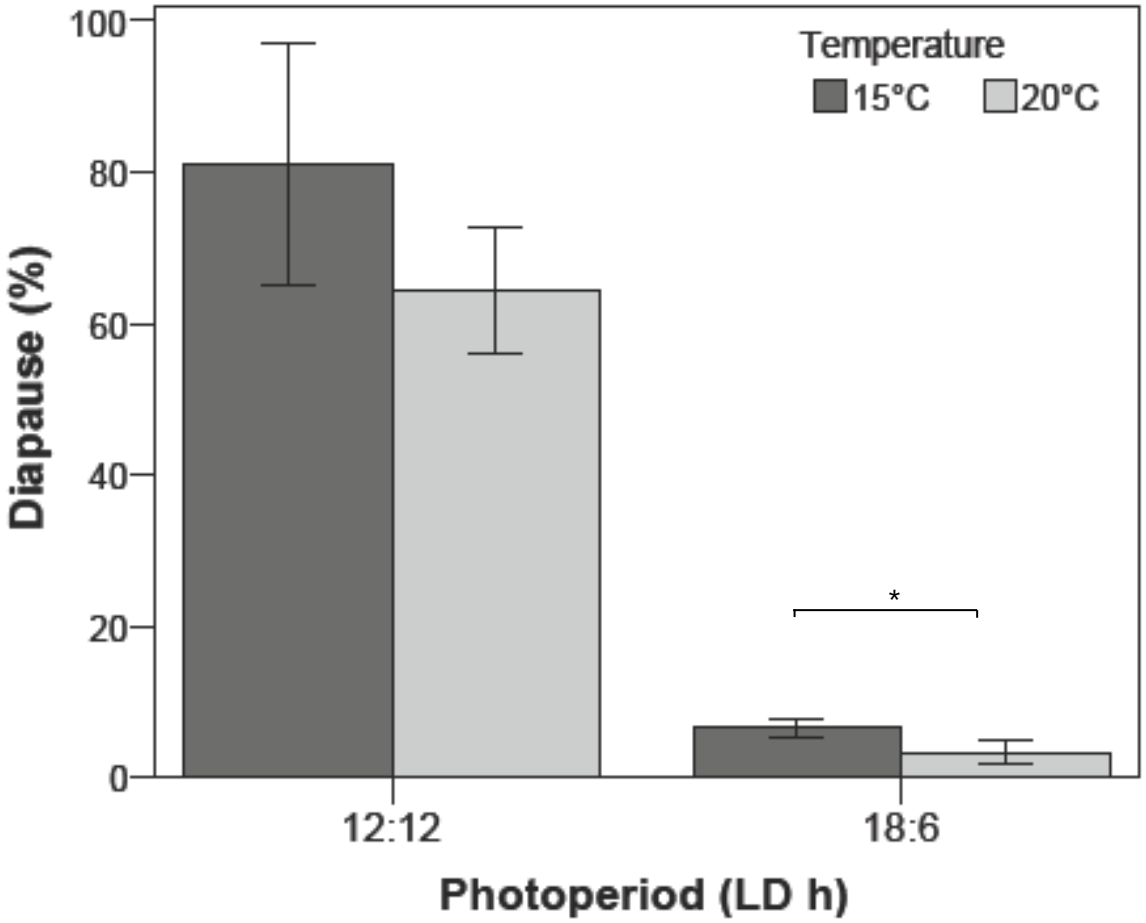


Figure 5.2 Mean percentage (±1 S. E. M.) diapause incidence in third-instar *C. vicina* larvae from parental populations acclimated to 15°C or 20°C under either a short-day (LD 12:12 h) or long-day (LD 18:6 h) photoperiod. * significant difference $P<0.05$. $n = 100 \times 4$ for each mean.

5.4.2 The effect of temperature on diapause duration in the laboratory

Diapause duration was clearly influenced by temperature. The time until pupariation was greatest for both D15 and D20 larvae at 5°C (50% pupariation after 64 d and 52 d, respectively) and shortest at 25°C (50% pupariation after 1 d for D15 and D20) (Table 5.1 and Figure 5.3).

Table 5.1 Statistical outputs to accompany Figure 5.3. The time taken for 10, 50 and 90% of diapause *C. vicina* larvae to pupariate when held at 5°C, 10°C, 15°C, 20°C or 25°C. Larvae are from parents acclimated to 15°C (D15) or 20°C (D20). Means marked with different letters and bold are significantly different, as determined by non-overlapping fiducial limits (percentile range is provided in brackets).

Temperature (°C)	**Pupariation (%)**	**D15** Mean (percentile range)	**D20** Mean (percentile range)
5	**10**	**40 (28-49) [a]**	**21 (20-22) [b]**
	50	**64 (57-71) [c]**	**52 (50-55) [d]**
	90	133 (118-147) [e]	103 (107 -124) [e]
10	**10**	**6 (6-7) [f]**	**2 (2-2) [g]**
	50	**21 (21-22)[h]**	**12 (12-13) [i]**
	90	**73 (70-78) [j]**	**63 (59-68) [k]**
15	10	1 (1-1) [l]	1 (1-1) [l]
	50	3 (2-3) [m]	2 (2-3) [m]
	90	5 (5-5) [n]	6 (5-6) [n]
20	10	1 (1-1) [l]	1 (1-1) [l]
	50	2 (2-2) [m]	2 (1-2) [m]
	90	**2 (2-2) [m]**	**3 (3-3) [o]**
25	10	1 (1-1) [l]	1 (1-1) [l]
	50	1 (1-1) [l]	1 (1-1) [l]
	90	2 (1-2) [l]	2 (1-2) [l]

Probit-analysis identified significant differences in the time taken to reach 10 and 50% pupariation at 5°C, and 10, 50 and 90% pupariation at 10°C, as determined by non-overlapping fiducial limits (Table 5.1). There were no significant differences in the diapause duration of D15 and D20 larvae at higher temperatures (15, 20 or 25°C), with the exception of time taken for 90% pupariation at 20°C (Table 5.1).

5.4.3 Climate data

Air temperature data between October 2009 and April 2013 Figure 5.4a) indicated that the warmest diapause period occurred in 2011-2012. This was also the only diapause period across years of study when temperatures exceeded 25°C, most frequently exceeded 15°C and 20°C, and least frequently fell below 0°C.

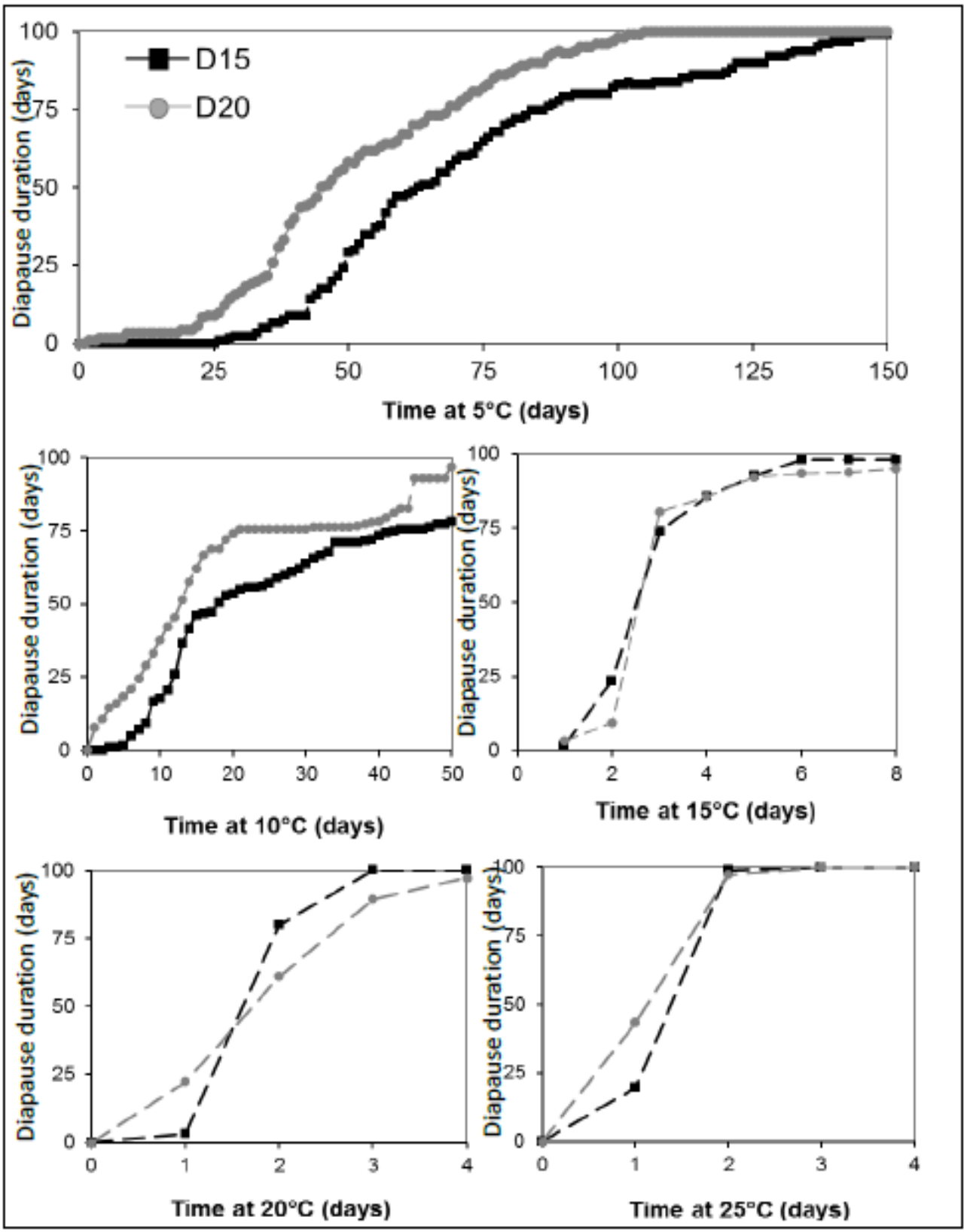


Figure 5.3 Cumulative pupariation rates for diapausing 3rd instar *C. vicina* larvae, when held under complete darkness at 5, 10, 15, 20 or 25°C. Larvae were from parental populations acclimated to either 15°C (D15; black line) or 20°C (D20; grey line). *n* = 18 × 10 individuals for each marker. Statistical differences are presented in Table 5.1.

Air temperatures between August and October (induction period) were lowest in 2012 (mean air temp of 12.61°C), and this was also the period when air temperatures only exceeded 20°C on 21 d. The warmest induction period was in 2011 (mean air temp of 14.20°C and 20°C exceeded on 29 d) (Figure 5.4a).

The lowest sustained temperatures between January and March (diapause termination/post-diapause quiescence) were experienced in 2013, with a total of 46 d experiencing temperatures below 0°C and a mean temperature of 2.72°C. The warmest post-diapause quiescence period was in 2012, with a mean temperature of 5.6°C and temperatures below 0°C only recorded on 3 d. For a summary of the frequency (number of days) and duration (number of hours) of events that key air temperature thresholds were exceeded refer to Table A1 and A2 in Appendices A.

Soil temperatures during diapause induction (August to September) were highest in 2009 and 2011, with mean temperatures of 15.07 and 15.15°C, respectively (Figure 5.4b). During this time, temperatures exceeded 15°C over 76 and 73 d, respectively. Temperatures were lowest during diapause induction in 2010 (mean soil temp of 13.92°C) when 15°C was exceeded over 66 d (Figure 5.4b).

Soil temperatures over November and December (diapause maintenance) were lowest in 2010, with the least number of days above 5°C (22 d) and most days below 0°C (12 d). Temperatures in the soil most frequently exceeded 5°C between January and March in both 2011 and 2012 (64 d for each).

Sustained soil temperatures below 0°C between January and March (post-diapause quiescence) were most frequent in 2010 (13 d) and least frequent in 2011 and 2013 (0 d for both). For frequency (number of days) and duration (number of hours) of events that key soil temperature thresholds were exceeded refer to Tables A3 and A4 in Appendices A.

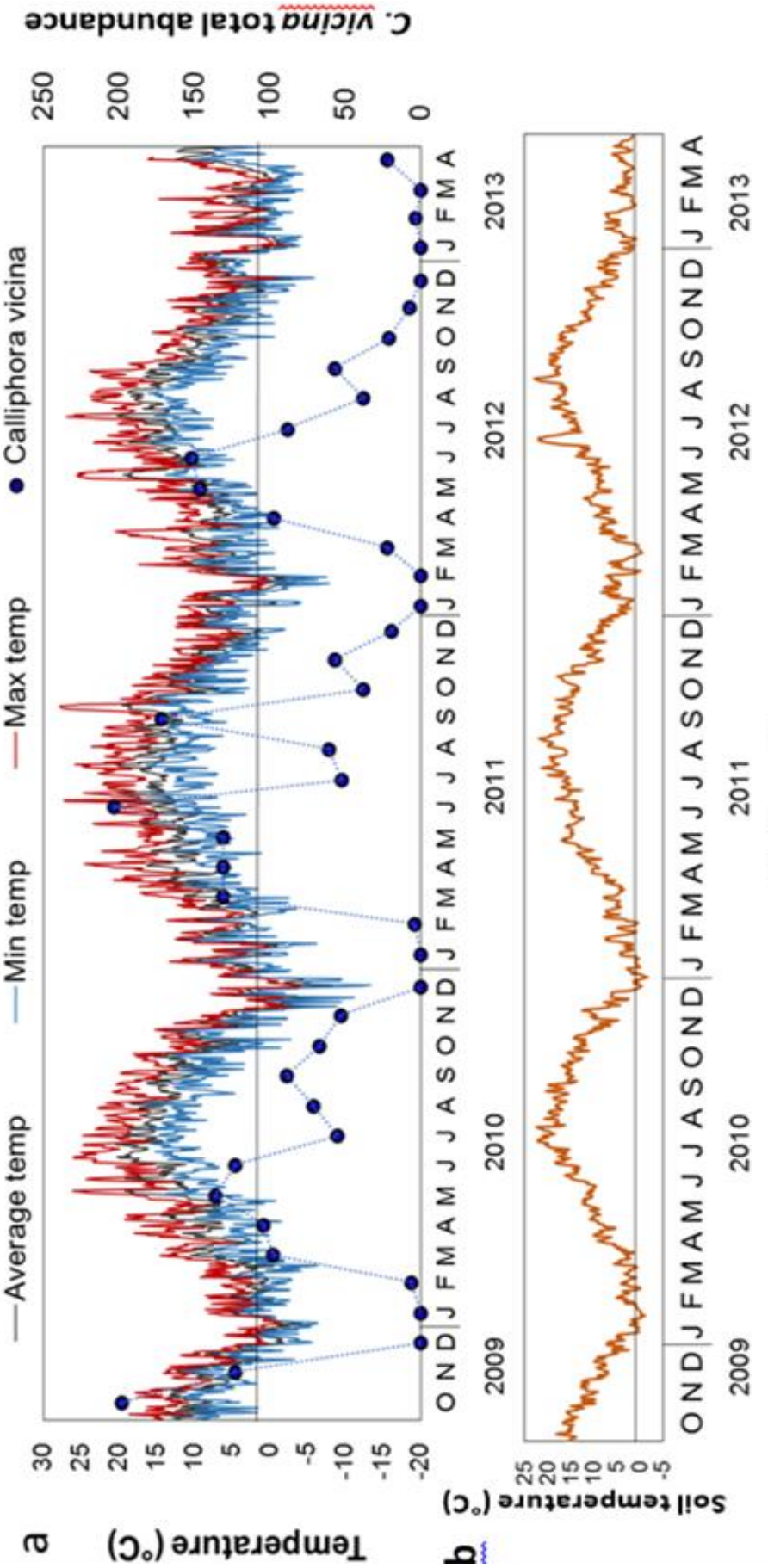


Figure 5.4 (**a**) The average (solid black line), minimum (solid blue line) and maximum (solid red line) daily air temperature, determined from hourly temperature measurements, from the 1st October 2009 to the 30th April 2013. The total abundance of adult *C. vicina* attracted to seven baited traps is represented by the dashed blue line and markers. On **b** is the average temperature (brown line) over the same period.

5.4.4 *C. vicina* phenology

The abundance of adult *C. vicina* followed a seasonal pattern of high abundance in summer and low abundance in winter (Figure 5.4a, 5.5a). In 2010, peak abundance was recorded in May (136 individuals), while in both 2011 and 2012 peak abundance was in June (203 and 152 individuals, respectively). These peaks were followed by a secondary, less prominent, peak in September in the years 2010 (89 individuals), 2011 (172 individuals) and 2012 (57 individuals).

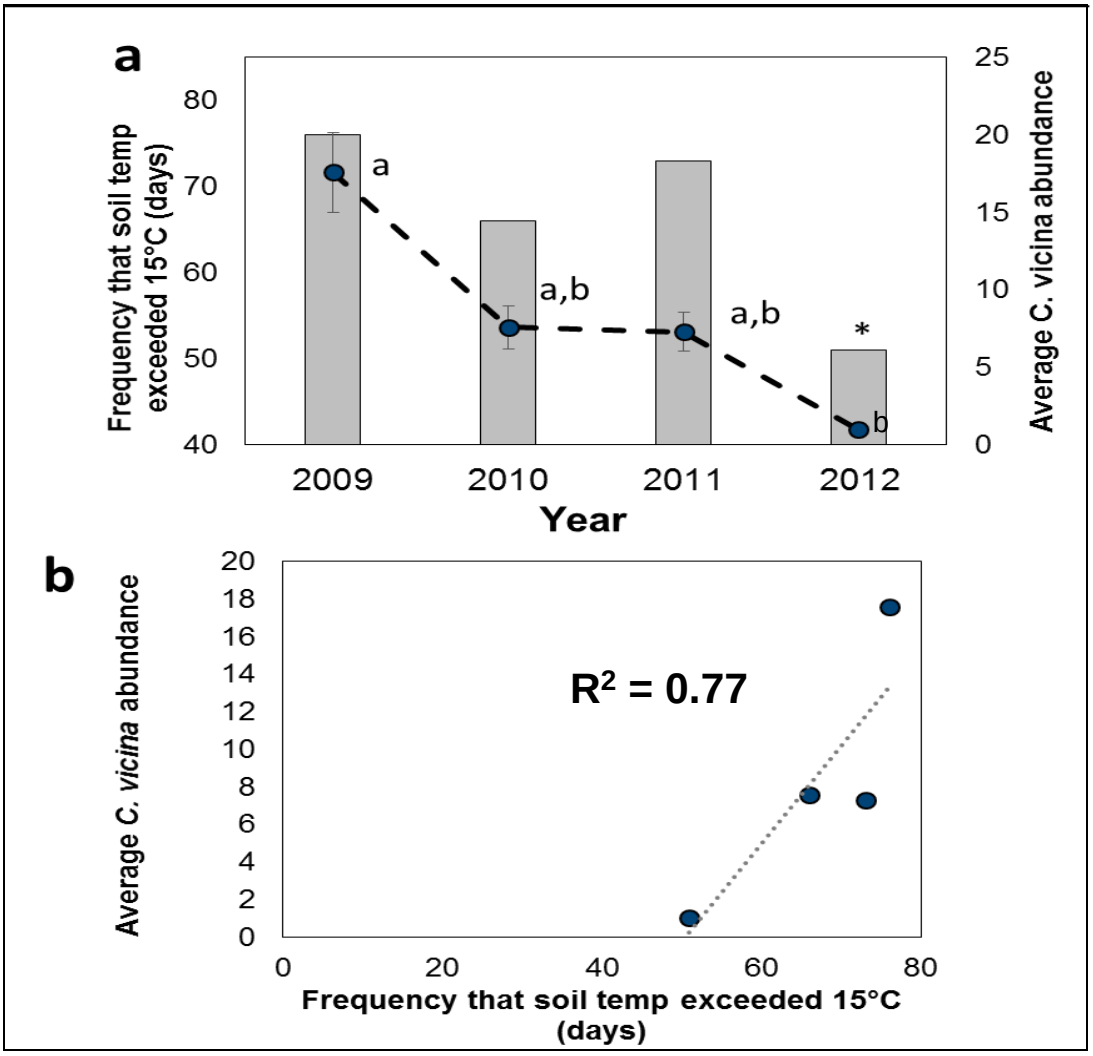


Figure 5.5 (**a**) Mean abundance (± 1 S. E. M.) of adult *C. vicina* (black line with blue points) in November between 2009 and 2012 against frequency (number of days) that soil temperature exceeded 15°C from 1st August to 31st October and (**b**)regression model and R^2 value for the same data. Significant differences (**a**) in adult abundance are marked with different letters. * *P*<0.05 (Bonferonni post-hoc).

Post-hoc analysis identified differences to be significant between 2009, the year when most active adults were recorded (average of 18 ± 5 individuals per trap), and 2012, the year when least adults were recorded (average of 1 ± 1 individuals) (Figure 5.5a and b). Adult activity in 2010 (8 ± 3 individuals) and 2011 (7 ± 2 individuals) were not significantly different from any other year (P>0.05).

The mean abundance of adults in November displayed a weak correlation to average air temperature in the month immediately preceding traps being placed in the field (R^2 = 0.31) (Figure A1 in Appendices A). There were also poor correlations between adult abundance in November and the number of days that air temperature exceeded 20°C between 1st August and 30th October (R^2 = 0.001) (Figure A2 in Appendices A), and days above 20°C in September (R^2 = 0.40) (Figure A3 in Appendices A).

There was, however, a strong correlation between the frequency that soil temperatures exceeded 15°C (Table A3 in appendices and Figure 5.5 b) in August, September and October and adult activity in November (R^2 = 0.77) (Figure 5.5 a and b). The frequency of soil temperatures exceeding 15°C was significantly different between years ($F_{3,368}$ = 7.1; P<0.001), with significantly fewer events in 2012 than in any other years (minimum of P<0.05 for all).

5.4.4.1 Adult activity in March (evidence of early diapause termination)

The number of adults recorded in March also varied significantly between years ($F_{3,28}$ = 7.01; P<0.01) (Figure 5.6a). Adult abundance was greatest in 2011 and this was significantly different from 2012 (P<0.05) and 2013 (P<0.001). Adult abundance was also significantly greater in 2010 than 2013 (P<0.05).

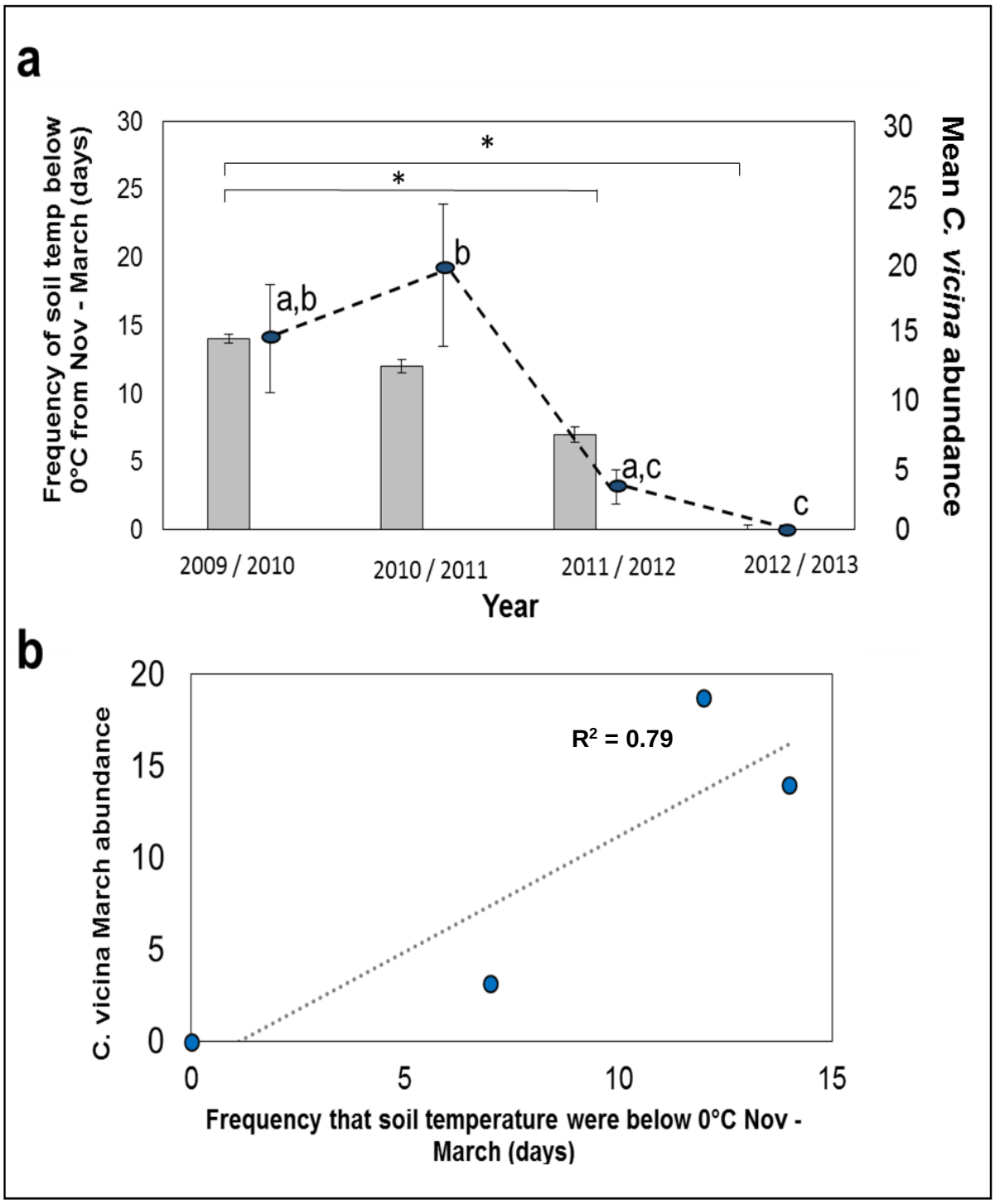


Figure 5.6 (**a**) Mean abundance (± 1 S. E. M.) of adult *C. vicina* (black line with blue points) in March between 2010 and 2013 against frequency (number of days) that soil temperature were below 0°C from November to March (**b**) Regression Model and R^2 for the same data. Significant differences (**a**) between adult abundances are marked with different letters and differences between average temperatures are marked by *, at $P<0.05$ (Bonferonni post-hoc).

Adult abundance in March displayed a weak correlation with average air temperature experienced in the month directly preceding traps being placed in the field (R^2 = 0.0009) (Figure A4 in Appendices A). There was also a weak correlation between average adult abundance in March and the duration that air temperatures exceeding 20°C between August and October (R^2 = 0.15) (Figure A5 in Appendices A) and, the duration that soil temperatures were above 5°C from January to March (R^2 = 0.21) (Figure A6 in Appendices A). There was no relationship between soil temperatures exceeding 15°C (zero days) from November to December and adult activity as temperatures did not exceed 15°C over this period.

There was, however, a strong relationship between the frequency of soil temperatures below 0°C between November and March and adult abundance in March (R^2 = 0.79) (Figure 5.6 a and b). The frequency of soil temperatures below 0°C was significantly different between years ($F_{s,604}$ = 5.40; P<0.001), with significantly fewer events over 2012/13 than 2009/10 (P<0.001) and 20010/11 (P<0.01) but not during the 2011/12 period (P=0.47).

5.4.5 Frequency and duration of temperatures above and below CT_{min} and chill coma

The number of days that temperatures were below the CT_{min} (0.9°C) for adult *C. vicina* were most in December in 2010 (22 d) and least in 2011 (5 d). In January, temperatures below the CT_{min} were most frequent in 2011 and 2012 (17 d for both) and least frequent in 2012 (12 d) (Figure 5.7).

For the month of February, temperatures below the CT_{min} were most frequent in 2010 and 2013 (22 d for both) and least frequent in 2011 (6 d). This information is presented in Figure 5.7 and in Table A5 in Appendices A.

The duration that temperatures were below the CT_{min} followed the same pattern as for frequency data. The duration of temperatures below the CT_{min} were greatest in 2010 for the month of December (434 h), 2009 for January (386 h) and in 2012 for February (216 h) (Figure 5.7 b and Table A5 in Appendices A).

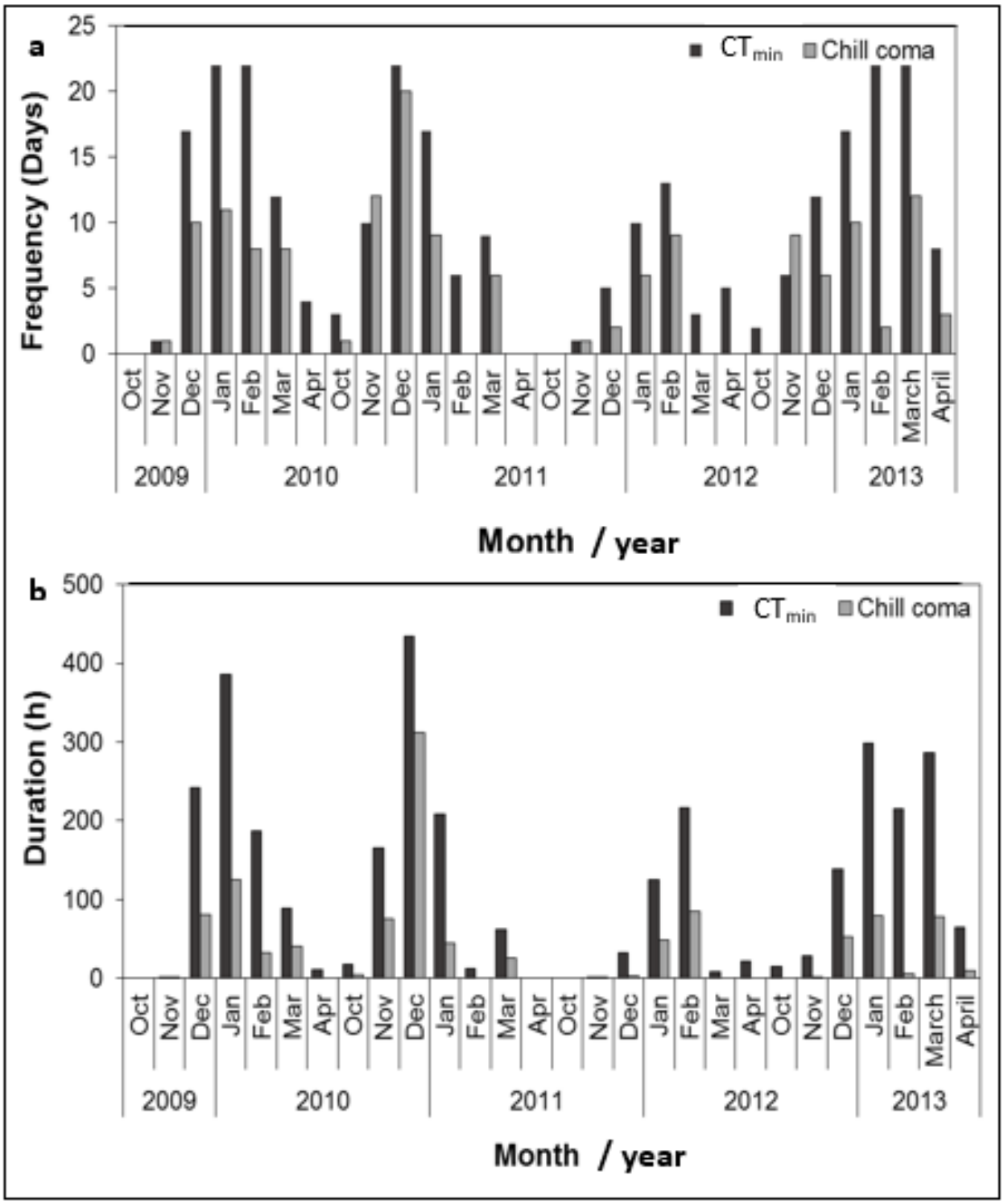


Figure 5.7 (**A**) Frequency and (**B**) duration of events where air temperature was below the CT_{min} (0.9°C) and chill coma (-1.7°C) for adult *C. vicina*. Recorded at an open field site on the University of Birmingham Campus, UK (52.4°N, 1.9°W) from Oct 2009 to April 2013. Frequency represents the total number of days when temperatures below the CT_{min} or chill coma were recorded. Duration is the total time that temperatures remained below the CT_{min} or chill coma over each month.

Additionally, temperatures below the CT_{min} and chill coma were frequently experienced in March in 2013 (22 d and 12 d, respectively) and less frequent over all other years (Figure 5.7 b). The duration of temperatures below the chill coma also followed the same pattern as for temperatures below the CT_{min} (-1.7°C). Temperatures below chill coma were most frequent in 2010 for the December months (20 d), 2010 for January (11 d) and 2012 for February (9 d) (Figure 5.7 a and b, and Table A6 in Appendices A).

5.4.6 Field trapping to monitor phenology of additional species

The monthly abundance of *L. sericata*, *S. carnaria*, *M. domestica*, *D. anilis* and *S. fenestralis* between October 2009 and April 2013 is summarised in Figure 5.8.

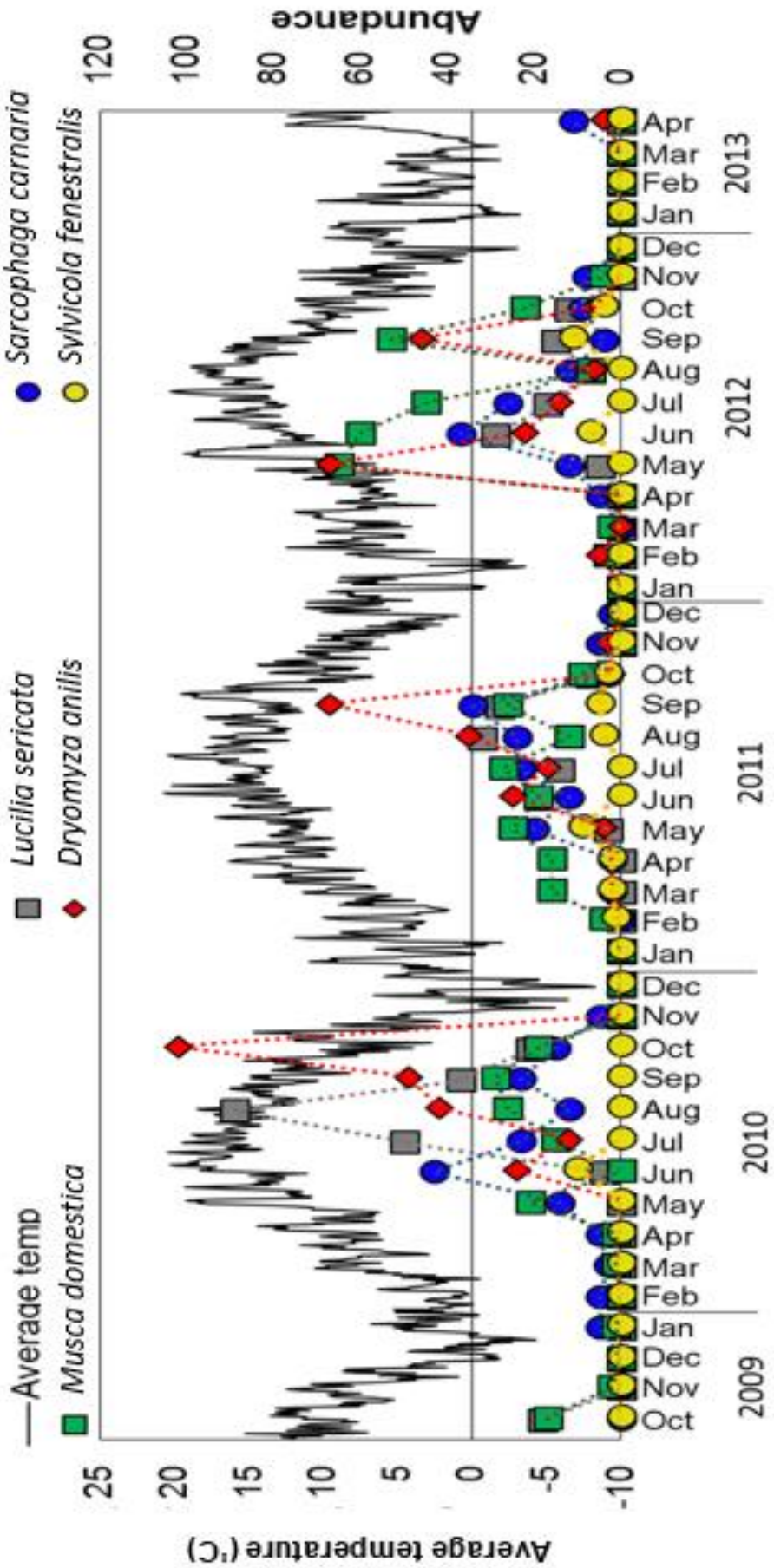


Figure 5.8 The average air temperature, determined from hourly temperature measurements, from the 1st October 2009 to the 30th April 2013 is represented by a solid black line. The abundance of five groups of adult Diptera attracted to seven baited traps are represented by different coloured markers joined by dashed lines. Temperature data were recorded in an open field and fly abundance monitored in deciduous tree coverage on the University of Birmingham campus, UK (52.4°N, 1.9°W).

5.5 Discussion

While phenological studies are often based on long-term datasets (Hassall et al., 2007; Richter et al., 2008; Graham-Taylor et al., 2009), short-term studies can also provide a valuable insight into the possible effects of climate change (Musolin, 2007; Sgolastra et al., 2011; Sgolastra et al., 2012). For insects, there will be positive responses to climate change, such as range expansions and the inclusion of additional yearly generations (Parmesan and Yohe, 2003; Musolin et al., 2010). There will also be negative responses, such as increased pest outbreaks and the rapid depletion of energy reserves (Sgolastra et al., 2011; Williams et al., 2012). This chapter confirms an additional response to climate change in that elevated temperatues have the capacity to delay entry into diapause. This phenomenon has also been reported in the pitcher plant mosquito, *Wyeomyia smithii*, and the fall webworm, *Hyphantria cunea* (Bradshaw and Holzapfel, 2001; Gomi et al., 2007).

For *C. vicina* populations in northern Britain (55°N), diapause is first induced when maternal adults detect a specific CDL of 14.5 h (Saunders, 1987). This maternal regulation of diapause is widespread at temperate locations (Mousseau and Dingle, 1991). While the specific CDL for the Birmingham strain is not known, Johnson (2013) established field-based cultures to confirm that adults active on the 9th September in 2009 and 2010 (day length of 12.45 h) produced predominantly diapause progeny (60 to 80% incidence). This was provided larvae were developed under diapause-permitting conditions (i.e low temperatures). Based on this knowledge, it was expected that adults would not be active from mid-October onwards, unless diapause-averting conditions were experienced (Vaz Nunes and Saunders, 1989; Vinogradova, 2011).

The presence of adults in November and December, and secondary yearly peaks in abundance in mid-September, confirm that diapause was being delayed by a proportion of the Birmingham population between 2009 and 2013 (Figure 5.4). Given that *C.vicina* have a generation time of 30 d (Johnson, 2013), it can be assumed that at least one extra

generation was included in 2009, 2010 and 2012 following detection of the CDL. In 2011, when adults were recorded in December, it is possible that two generations were completed following first detection of the CDL in September (Figure 5.5 and Table A1 in Appendices A).

The environmental factors responsible for the observed delay in the programming of diapause can be explained using a combination of *in-situ* measurements and laboratory based indices. Previous studies have confirmed that temperatures must remain suitably low during diapause induction and maintenance, or diapause incidence is reduced or averted completely (Tauber et al., 1986; Vaz Nunes and Saunders, 1989; Tachibana and Numata, 2004b; Vinogradova, 2011). Based on this knowledge, there is no evidence to suggest that adults are encountering diapause-aborting conditions. Air temperature in autumn and winter rarely exceeded 25°C (only on 6 d between August and October, 2011), the threshold temperature above which diapause is not induced (Table A1 in Appendices A) (Vinogradova, 2011). Additionally, no relationship was detected between air temperature exceeding 20°C and a delay in the timing of diapause (Figures A2 and A3 in Appendices A). Using measurements of diapause incidence under different parental culturing temperatures (Figure 5.2), it was expected that between 60 and 80% of the larval population would enter diapause under late-autumn temperatures experienced *in*-situ. This is provided that larvae experienced temperatures below 15°C (Vaz Nunes and Saunders, 1989).

The evidence presented in this chapter suggests that the delay in the timing of diapause was due to high soil temperatures experienced by L3 larvae. The diapause duration times, presented in Figure 5.4, clearly illustrate that all larvae (regardless of parental temperature) complete diapause within 8 d when held at 15°C, compared to over 100 d at 5°C (see also, Vaz Nunes and Saunders, 1989). This understanding, combined with temperature recordings which show that soil temperatures regularly exceeded 15°C between August and October (51 to 76 d; see Table A3 in Appendices A), suggest that L3 larvae were encountering diapause averting temperatures. This was further substantiated by a strong correlation

between the frequency of soil temperatures above 15°C and the abundance of adults in November (Figure 5.5a and b).

Higher larval temperatures are associated with increased metabolism and the rapid depletion of the energy reserves (Bale and Hayward, 2010; Hahn and Denlinger, 2011). Under higher temperatures, it is advantageous to abort diapause, complete an additional generation, accumulate necessary energy reserves and re-attempt diapause. This is an adaptive response that increases the likelihood of overwinter survival rather than attempting to overwinter without the reserves in place that are required for winter survival (Hahn and Denlinger, 2011). For some univoltine species, such as the solitary bee, *O. lignaria*, it is not possible to avert diapause, even under higher temperatures. In this species, warmer winters are associated with increased mortality (Sgolastra et al., 2011). Larval sensitivity to higher temperatures could therefore prove to be a positive adaptive trait under future climatic conditions. In species capable of averting diapause increasing the likelihood of winter survival, by delaying entry into diapause until temperatures are suitably low to ensure winter survival.

In comparison to *C. vicina*, the phenology of *L. sericata* was more consistent between years, with adults consistently active until October (Figure 5.8). For *L. sericata*, diapause is firstly induced by maternal detection of a CDL and then secondly reinforced by photoperiod-sensitive larvae (Tachibana and Numata, 2004b). Photoperiod sensitivity in the larval stage is likely to mitigate exposure to diapause-aborting temperature cues, but, this has not been confirmed experimentally. Furthermore, larvae must experience temperatures of 20°C or above to avert diapause (Tachibana and Numata, 2004a), making them less susceptible than *C. vicina* to temperature fluctuations. These findings suggest that overwintering life stages of *L. sericata* are less susceptible to climate change than *C. vicina*. Evidence that these two closely related species could respond very differently to warmer autumn temperatures, reinforces the complexity in predicting how species will respond to climate change. Little is known about the physiology of *S. carnaria*, *D. anilis* and *S. fenestralis* and it is not known

how any of these species survive the winter period. All three species displayed a high degree of variation in their yearly phenology. Yearly fluctuations were generally consistent with changes in temperature. Adult activity was maintained until latest in the year in 2011, the warmest year of the study period (Figure 5.8). This further substantiates the expectation that warmer autumn and winter temperatures will disrupt the overwintering life stages of the majority of temperate insects (Bale and Hayward, 2010).

The relationship between March activity (diapause termination) and prevailing environmental conditions appears more complex than the relationship between the environment and the induction of diapause. It was hypothesised that temperatures experienced during the sensitive stage (August to October), as well as subsequent developmental stages (diapause maintenance and post-diapause quiescence) could affect the time of spring emergence (Bale and Hayward, 2010; Hahn and Denlinger, 2011). Within the laboratory, diapause duration was shortened following adult acclimation to a higher temperature (Figure 5.3; McWatters and Saunders, 1998). However, no *in-situ* relationship was detected between parental culturing temperature in autumn/winter (August to October) and the duration of diapause (Figure A5 in, Appendices A). This does not conflict with adult temperature having an influence on the diapause phenotype, such as the cross generation effect of cold hardiness presented in Chapter 2. A relationship did exist, nonetheless, between the frequency that soil temperatures were below 0°C from November to March (diapause maintenance/post-diapause quiescence) and spring emergence. A greater frequency of temperatures below 0°C was associated with earlier spring emergence. This *in-situ* response to low temperature does not correspond to laboratory indices, were lower temperatures are associated with a longer diapause duration (Figure 5.3), as has been observed in a previous studies on *C. vicina* (Johnson, 2013).

It is likely that diapause termination is a complex response to numerous environmental variables, including the timing of diapause induction. Under laboratory conditions, 50% of larvae completed diapause in under 64 d when held at 5°C (Figure 5.3). *In-situ* average soil

temperatures during diapause maintenance (November to December) ranged between 3.2°C (2010) and 6.8°C (2011), while post-diapause quiescence (January to March) temperatures ranged from 3.3°C (2011/2013) to 5.4°C (Figure 5.4a). It is realistic to assume that under these *in-situ* conditions, with temperatures close to 5°C, the duration of diapause would be close to 64 d. This assumption is substantiated by observational evidence of a complete absence of adults for two months over each year of the study (Figure 5.4). It is therefore possible that the spring emergence of adult *C. vicina* is predominantly controlled by the timing of diapause induction, with diapause lasting for approximately two months under the similar soil temperatures experienced overall years of the study.

The emergence of adults in February 2013 was followed by a subsequent absence of adults in March (Figure 5.4a). This coincided with a greater frequency of temperatures below adult CT_{min} and chill coma (22 d and 12 d, respectively) than over the same period during any other year (Figure 5.7 a and b). These findings reinforce the use of CT_{min} and chill coma as ecologically relevant measures of cold tolerance, beyond which individuals are unable to relocate to more favourable environments, locate food, avoid predation, or even reproduce (Hughes et al., 2010; Macmillan and Sinclair, 2011; Coombs and Bale, 2013; Everatt et al., 2013).

In general, adult activity ceased in November, while the duration and frequency of temperatures below the CT_{min} and chill coma were greatest in January (Figure 5.7 a and b). Adults were even absent during January in 2011, when the duration of temperatures below the CT_{min} (10 d) was lower than any other year (22, 17 and 17 d for 2010, 2012 and 2013, respectively) and very similar to other times of the year when adults were active, such as March 2011 (9 d and 14 ± 4 individuals). This suggests that the absence of adults over the coldest parts of the year is not a direct response to low temperatures, but is part of the anticipatory response of diapause, which has evolved to synchronise behavioural and physiological adaptations with periods of acute and chronic winter cold (Tauber and Tauber, 1976).

5.6 Conclusions

The relationship between temperature and diapause is far more complex than increased temperatures simply blocking the ability to enter diapause. For *C. vicina* and *L. sericata*, adult temperature has the capacity to modify the duration and incidence of diapause (Saunders, 1987; Tachibana and Numata, 2004b), and the cold tolerance phenotype (Chapter 2). Evidence presented in this chapter indicates that the induction of diapause in *C. vicina* is being delayed, beyond the maternal detection of the CDL, due to temperatures experienced by third-instar larvae. This response was identified using a combination of field observations, laboratory indices and environmental data. The timing of diapause termination appears to be more complex, but could in part be influenced by the timing of diapause induction in late-autumn/early spring. As temperatures continue to increase (by up to 4°C by the end of this century; IPCC, 2013) the photoperiod and temperature cues utilised by the majority of temperate insects to induce diapause will become increasingly disrupted (Bale and Hayward, 2010). This has the potential to delay diapause until later in the year. However, the results presented in this chapter indicate that even two closely related species, *L. sericata* and *C. vicina*, respond very differently to warmer autumn temperatures. Responses to climate change will ultimately be varied and species-specific and therefore difficult to predict.

The absence of adults during the coldest periods of the year appears to be a pre-emptive response. This was confirmed by an absence of adults in January 2011. During January, temperatures were usually below thermal activity thresholds on a regular basis. However, in 2011 the temperatures in January were higher than other years, and very similar to periods of the year when adult activity was regularly maintained. The absence of adults over this time, when activity could have been maintained, confirms that diapause in *C. vicina* is an anticipatory response, which has evolved to synchronise behaviour and physiology with the changing seasons.

CHAPTER 6

DOES ARTIFICIAL SELECTION FOR DIAPAUSE INCIDENCE MODIFY THE COLD HARDINESS PHENOTYPE?

6.1 Abstract

For the majority of temperate insects, the initiation of diapause is associated with an increase in cold-hardiness. However, it is unclear whether diapause is a prerequisite for enhanced cold tolerance, or if the two phenomena are induced independently, although over a similar time scale, following detection of similar environmental cues. In this chapter, the effects of environmental stimuli were disentangled from the diapause response through selection for diapause (DS) and non-diapause (NDS) strains. All under photoperiodic (LD 12:12 h) and temperature (under 20°C) conditions known to induce diapause and enhance cold hardiness. This was performed in larvae produced from adults acclimated to 15°C (DS15 and NDS15, respectively) and 20°C (DS20 and NDS20, respectively) under a short-day photoperiod (LD12:12 h).

Diapause incidence rose from between 55 and 65%, to 82% for the F2 generation of the DS15 line and 72% for the DS20 line. Incidence fell to 24% by the F2 generation of NDS15 and to 0% for NDS20. The changes in diapause incidence coincided with alterations in supercooling points (SCPs) and the ability to tolerate chronic cold exposure at -8°C (LT_{50}).

In the F2 generation, the mean SCPs of DS20 larvae were significantly lower than for NDS20 larvae. In the F5 generation, the mean SCPs of DS15 larvae were significantly lower than for NDS20 larvae. A similar trend was observed in the time taken to induce 50% mortality (LT_{50}) following -8°C exposure, with increased cold tolerance in the F2 generation DS15 and DS20 larvae compared to NDS15 and NDS20 larvae.

The establishment of selection lines also allows the magnitude of heritability (h^2) in the diapause incidence and supercoiling capacity to be investigated. Heritability was low for high diapause incidence (h^2 = 0.23), which suggests that high diapause incidence is a well conserved trait closely associated to individual fitness. Conversely, there was high heritability in supercoiling capacity (h^2 = 0.54 and 0.74 for high and low incidence selection lines,

respectively). This high heritability indicates that supercoiling capacity is not a well conserved trait. This could be explained in part by the low evolutionary pressure to maintain a high supercoiling capacity, with individual's *in-situ* rarely experiencing temperatures approaching their SCPs.

This chapter confirms a close association between diapause incidence, supercooling capacity and cold hardiness, and suggests that diapause is a pre-requisite for implementing seasonal increases in cold hardiness. It also confirms that diapause phenotype is closely associated with individual fitness.

6.2 Introduction

Insects at high latitudes often display predictable patterns in growth, reproduction and dormancy, associated with the changing seasons (Tauber et al., 1986). A common characteristic of insect seasonality is the occurrence of diapause, a pre-emptive response to adverse environmental conditions (Denlinger, 1991). For many species, it is unclear whether diapause is a prerequisite of cold hardiness (Goto et al., 2001; Khodayari et al., 2013). It is possible that seasonal cold hardiness and diapause are induced independently, albeit at the same time, through changing environmental conditions (Ring, 1972; Tanaka and Zhu, 2008). The evidence in support of a shared relationship is stronger than the evidence against. Adults of the firebug, *Pyrrhocoris apterus*, exhibit a stronger supercooling capacity when exposed to short-day photoperiods and low temperatures, but only when in diapause (Hodkova and Hodek, 1994, 1997). Diapausing pupa of the flesh fly, *Sarcophaga crassipalpis*, are more cold tolerant than non-diapause counterparts (Lee and Denlinger, 1985), as has been observed in diapausing adults of the Coleopteran, *Aulacophora nigripennis* (Watanabe and Tanaka, 1998) and numerous other species (Mansingh, 1974; Pullin et al., 1991; Manrique et al., 2012; Murata et al., 2013), including *Calliphora vicina* (Saunders and Hayward, 1998; Johnson, 2013; Chapter 2).

However, in the Coleopteran, *Dendroides canadensis*, a short-day photoperiod increases cold hardiness but does not induce diapause (Horwarth and Duman, 1983), while for the European corn borer, *Ostrinia nubilalis*, a short-day photoperiod induces diapause but does not increase cold hardiness (Denlinger, 1991). Also, there is no difference in cold tolerance between diapause and non-diapause larvae of the green bottle, *Lucillia sericata* (Ring, 1972). Thus, the relationship appears species-specific. It is therefore possible that (1) diapause and cold-hardiness represent a synonymous stress response mechanism that has evolved to post pone development and heighten cold tolerance, (2) cold hardiness has arisen as a by-product of diapause or (3) the two phenotypes are induced by independent stimuli over the same timeframe.

In the blow fly, *C. vicina*, diapause is induced through the inhibition of ecdysteroid hormones following maternal detection of the CDL (Richard and Saunders, 1987). Larval diapause is associated with an increased supercooling capacity (see Figure 2.2 in Chapter 2) and heightened cold hardiness (Figure 2.4), which is further modified by parental acclimation temperature (Figures 2.3 and 2.4). The intensity of diapause is also modified by parental acclimation temperature, with higher temperatures reducing diapause incidence and shortening its duration (McWatters and Saunders, 1998). A similar response has been identified in the mosquito, *Aedes togoi*, and parasitoid wasp, *Trichogramma evanescens* (Anderson, 1968; Zaslavski and Umarova, 1990).

C. vicina also displays a latitudinal variation in diapause-associated cold hardiness. Strains from Edinburgh, Scotland (55°N), display 40% survival following exposure to -8°C for 12 d, while strains from Barga, Italy (44°N) encounter total mortality following 7 d exposure at the same temperature (Saunders and Hayward, 1998). Similarly, diapausing larvae from the Edinburgh strain encounter less mortality following -4°C and -8°C exposure than strains from Nallikari, Finland (65°N) (Saunders and Hayward, 1998). The increased cold hardiness of the Edinburgh strain is a likely response to being exposed to variable winter temperatures, while the Finland strain is protected from above ground temperature fluctuations due to extensive

winter snow cover. Latitudinal gradients in cold hardiness often coincide with changes in diapause incidence. For example, the migratory locust, *Locusta migratoria,* exhibits a latitudinal gradient in genetic make-up, diapause intensity and diapause-associated cold hardiness (Tanaka and Zhu, 2008). Northern populations of *L. migratoria* (47°N) exhibit an obligatory diapause, while for southern populations (40°N to 19°N) it is a facultative process. The incidence of diapause increases as adult temperature is reduced and photoperiod is shortened (Tanaka and Zhu, 2008). Cold hardiness follows the same latitudinal trend as diapause incidence, with a pole-ward increase in cold tolerance providing positive adaptive traits to local environmental conditions.

The close association between diapause incidence and cold hardiness further reinforces the link between the two phenotypes. Still, it remains possible that they are induced by independent stimuli over the same timeframe. A novel approach to disentangle the relationship between diapause, cold hardiness and environmental cues is to compare the cold hardiness capabilities of diapause and non-diapause life stages that have experienced identical diapause-inducing conditions. For example, a proportion of *C. vicina* larvae, even after experiencing diapause-inducing conditions (as adults and larvae), will always avert diapause and instead favour continued development. For adults acclimated to 20°C, under a diapause inducing CDL, 20-30% of larvae in the subsequent population will avert diapause (Johnson, 2013). This is likely to be an adaptive bet-hedging strategy, with variation in offspring phenotypes guaranteeing that at least some individuals will be well-suited to future climatic conditions. A similar bet-hedging strategy has been associated with diapause in a number of species, including the bushcricket, *Ephippiger ephipigger* (Hockham et al., 2001) and the fruit fly, *Drosophila melanogaster* (Schmidt et al., 2005).

This bet-hedging phenomenon provides an opportunity to further investigate the relationship between diapause and cold hardiness. It is possible to use artificial selection lines to establish high and low diapause incidence lines under diapause-inducing conditions (Henrich and Denlinger, 1982; Gilkeson and Hill, 1986). Previous studies have used this approach to

produce low-incidence strains of the predatory midge, *Aphidoletes aphidimyza*, for establishment as a biocontrol agent (Gilkeson and Hill, 1986), to investigate circadian clock mechanisms in *C. vicina* (Saunders and Cymborowski, 2003) and to investigate the relationship between diapause incidence and duration in the flesh fly, *Sarcophaga bullata* (Henrich and Denlinger, 1982). The primary aim of this this chapter is to use high and low diapause incidence lines to disentangle the role played by environmental conditions experienced during diapause induction (adults for *C. vicina*) from diapause initiation and the associated increase in cold hardiness. Through the establishment of selection lines it is possible to determine whether levels of cold tolerance (determined through LTs and SCPs) are associated with the diapause incidence of a population. If there is a relationship between the two phenotypes then it is expected that populations with increased diapause intensity will also display increased levels of cold hardiness.

The establishment of selection lines also allows the magnitude of genetic variation in the diapause phenotype to be investigated. Selective breeding has been used in previous studies on mice (*Mus domestica*) and *Drosophila melanogaster* to determine the genetic basis of particular phenotypes (in these examples defensive behaviour and upper temperature thresholds, respectively) (Gilchrist and Huey, 1999; Gammie et al., 2006).

The phenotypic variation of a particular trait is the result of both environmental and biological factors. It is possible to determine how much of the variation is due to biological factors through determination of the heritability of the phenotype (Swallow et al., 1998). A phenotype with high heritability indicates a strong resemblance between generations, while a low heritability suggests that environmental factors and individual fitness may be more important in the expression of the phenotype (Lynch and Walsh, 1998).

Heritability can be divided into narrow-sense heritability and broad-sense heritability (Lynch and Walsh, 1998). Broad-sense heritability involves all of the genetic contributions to a population's phenotype (including effects that may be due to dominance and epistasis) and is usually associated with asexual reproduction, while narrow-sense heritability captures only

the proportion of genetic variation due to additive genetic values (Lynch and Walsh, 1998). The response to artificial selection relies on additive genetic variation; for this reason previous selection studies have focused solely on narrow-sense heritability (Lynch, 1980; Swallow et al., 1998; Gilchrist and Huey, 1999; Morrow and Gage, 2001; Gammie et al., 2006). A well-established approach in determining the narrow-sense heritability of a phenotype is to calculate the realised heritability (h^2), this is the degree to which a trait can be enhanced through selective breeding (Lynch and Walsh, 1998).

The ecological significance of the h^2 value is that it provides information on how easily a trait can be passed between generations, possibly leading to the increased presence of a positive adaptive trait and ultimately to evolutionary adaptation (Hoffmann et al., 2005). In this chapter I determine the h^2 of diapause incidence and cold tolerance (following the approach of Gilchrist and Huey, 1999). Both of these traits display a high degree of variation within a population so it is assumed that they will have high h^2 values.

It is hypothesised that (1) if diapause and cold-hardiness are mutualistic stress response mechanisms, the low incidence line will be less cold hardy than the high incidence line. I also hypothesise that (2) if the two phenotypes are induced by independent stimuli, the low incidence line will retain the same levels of cold tolerance as the high incidence selection line. The heritability of diapause intensity is also investigated and it is hypothesised that (3) diapause incidence and cold tolerance are both heritable responses that can be selected for over multiple generations and will have high h^2 values. Selection lines were maintained over several generations with parental populations acclimated to either 15°C or 20°C. Establishing selection lines under two parental temperatures also allowed the cross generational effect of temperature on cold tolerance, as presented in Chapter 2, to be further investigated over multiple generations.

6.3 Materials and methods

6.3.1 Experimental cultures

C. vicina used in this chapter were originally sourced from the University of Birmingham campus, Birmingham, UK (52.4°N, 1.9°W) in 2009 using olfactory traps (Figure 2.1; Hwang and Turner, 2005). Laboratory cultures were then regularly replenished (every 3 months) with wild-caught individuals. For the maintenance of stock cultures please refer to Chapter 2, section 2.3.1.

6.3.2 Diapause incidence

Selection lines were originally established from the F0 generation adult cultures established under a short-day photoperiod (LD 12:12 h) at either 15°C or 20°C. Under short-day photoperiods, parental cultures give rise to predominantly diapausing progeny, which are identified as L3 larvae not pupariating by d 30 post-oviposition (Saunders, 1987).

On d 30 post-oviposition, F0 generation L3 larvae were separated into individuals which had pupariated (non-diapause) and those that were still L3 larvae (diapause). Pupae were used to establish the non-diapause selection (NDS) line, while larvae were used to establish the diapause selection (DS) line (Saunders, 1987). Before and after separation into DS and NDS lines, larvae and pupae were held at 11°C in complete darkness until the time of adult eclosion. Newly eclosed adults were then used to establish the F1 population. NDS and DS F1 adults were held at the same temperature as their F0 parents, either 15°C (NDS15 and DS15) or 20°C (NDS20 and DS20). Adult cultures received sugar and water *ad libitum* and pig liver as a source of protein and site of oviposition as described in Chapter 2, section 2.3.1.

Progeny from the F1 generation adults were again separated into pupae and larvae on d 30 post-oviposition. For the DS line, pupae were discarded and only larvae used to establish the F2 generation. For the NDS line, larvae were discarded and only pupae were used to

establish F2 generation. This procedure was followed until the F7 generation for DS15 larvae and until the F5 generation for NDS15 larvae and until the F2 generation for DS20 and NDS20 selection lines.

6.3.3 Supercooling capacity

Supercooling capacity was determined through identification of individuals' SCPs at a cooling rate of 0.5°C min^{-1}, as described in Chapter 2 section 2.3.3. Supercooling capacity was determined for the F1 generation DS15 and DS20 larvae following acclimation to 5°C for three months from the time of mass wandering (d 15 post-oviposition). Three months acclimation to 5°C was selected as a temperature likely to be experienced by overwintering larvae in Birmingham, UK. There were *n* = 16 individuals for both treatments.

The supercooling capacity was also determined for the F2 generation DS20 and NDS20 larvae and F5 generation DS15 and NDS20, all of which were held under complete darkness at 11°C from the time of egg oviposition (d 0 post-oviposition). There were *n* = 8 for DS15, 29 for NDS15, and 30 for DS20 and NDS20.

6.3.4 Lethal time (LT)

Chronic cold tolerance was determined as the Lethal Time (LT) taken for 10% (LT_{10}) and 50% (LT_{50}) mortality following prolonged exposure to -8°C. The methodology followed that outlined in Chapter 2, section 2.3.4. Briefly, groups of 10 L3 larvae were placed in 10 ml glass vials containing 1 cm of sawdust, held in a programmable incubator at -8°C, and removed at 3 d intervals over a period of 18 d. Removed vials were held at 11°C in darkness for 24 h then at 20°C until eclosion. Larvae failing to eclose after 21 d were considered dead. Controls were maintained at 11°C for the duration of each experiment. A probit analysis was then used to estimate the time taken to kill 10% (LT_{10}) and 50% (LT_{50}) of the population. Methods used to investigate the LT are based on previous approaches (Saunders and

Hayward, 1998; Hart et al., 2002; Hughes et al., 2009, 2010). There were *n* = 60 individuals per treatment.

This was followed for the F1 generation DS15, DS20, NDS15 and NDS20 larvae and the F5 generation DS15 and NDS15. All larvae were held in complete darkness at 11°C from the time of oviposition until experimental use.

6.3.5 Realised heritability (h^2)

To estimate heritability in diapause incidence for the population, the mean diapause incidence values of the F1 generation were subtracted from the mean of the parental generation to determine additive genetic variance, represented as Var (A). To determine the proportion of phenotypic variation, represented as Var (P), the mean diapause incidence value from the F2 generation were subtracted from the mean of the parental generation. The h^2 was then calculated using the following equation (Lynch and Walsh, 1998):

$$h^2 = \frac{Var\ (A)}{Var\ (P)} \qquad \textbf{Equation 6.1}$$

Estimates for the h^2 of diapause incidence were determined for all treatments (DS15, DS20, NDS15 and NDS20). The h^2 for supercoiling capacity was calculated using the same equation, for the mean SCPs for the F0, F1 and F2 generation.). h^2 values range between 0 and 1. A value below 0.15 indicates very low heritability in a phenotype and suggests that the trait is closely associated to individual fitness while values exceeding 0.40 are highly heritable (Lynch and Walsh, 1998).

6.3.6 Statistical analysis

SCP data analysis was undertaken using SPSS (v. 20.0, IBM, New York, USA). Data were analysed using GLMs. Significant differences between groups were identified using the Bonferroni *post-hoc* test with an alpha threshold of 0.05.

LT data were analysed using the statistical package Minitab 15 (Minitab, Coventry, UK). A probit analysis, based on Finney (1971), was used to determine the LT_{10} and LT_{50} by identification of non-overlapping upper and lower percentiles (± 95% fiducial limits) as in Hart et al. (2002).

6.4 Results

6.4.1 Diapause incidence

The incidence of diapause in the F0 generation was between 55 and 65%. For the DS15 line, diapause incidence had risen to 81% by the F2 generation and 100% by the F5 generation. Incidence then declined slightly in the F6 and F7 generations, but remained at 89% or above (Figure 6.1).

6.4.2 F1 generation

6.4.2.1 Supercooling capacity

The supercooling responses of F1 generation DS15 and DS20 larvae following three months acclimation to 5°C were not significantly different ($F_{1,26}$=1.02, *P*=0.32) (Figure 6.2).

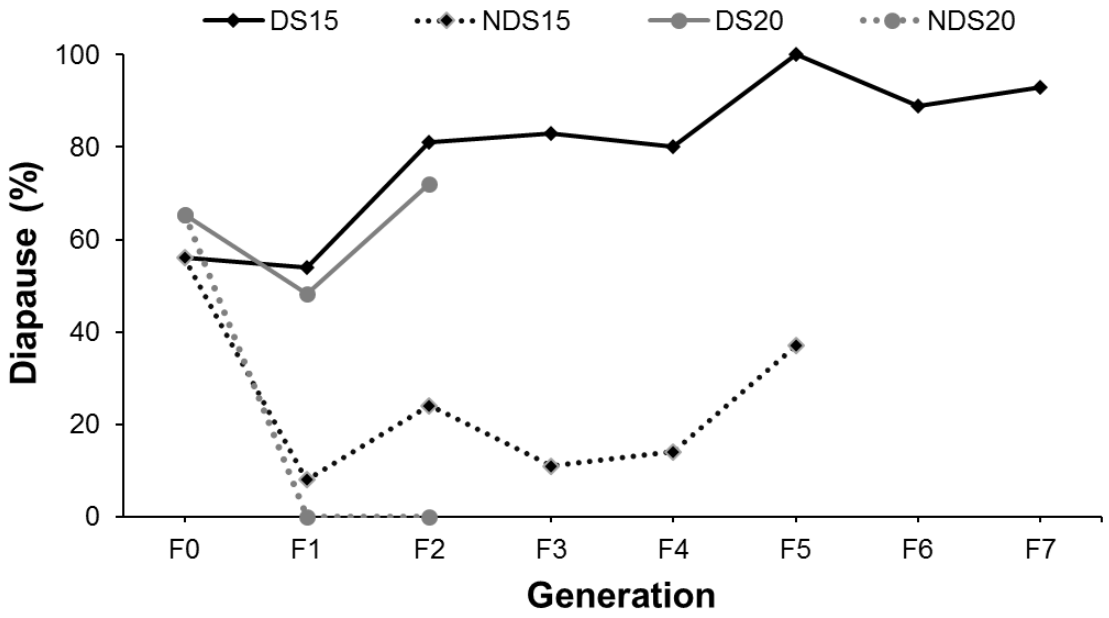


Figure 6.1 Selection for high (solid lines) and low (dashed lines) incidence of larval diapause in *C. vicina* larvae produced from adults acclimated to 15°C (DS15 and NDS15, respectively) or 20°C (DS20 and NDS20, respectively) under a short-day photoperiod (LD 12:12 h).

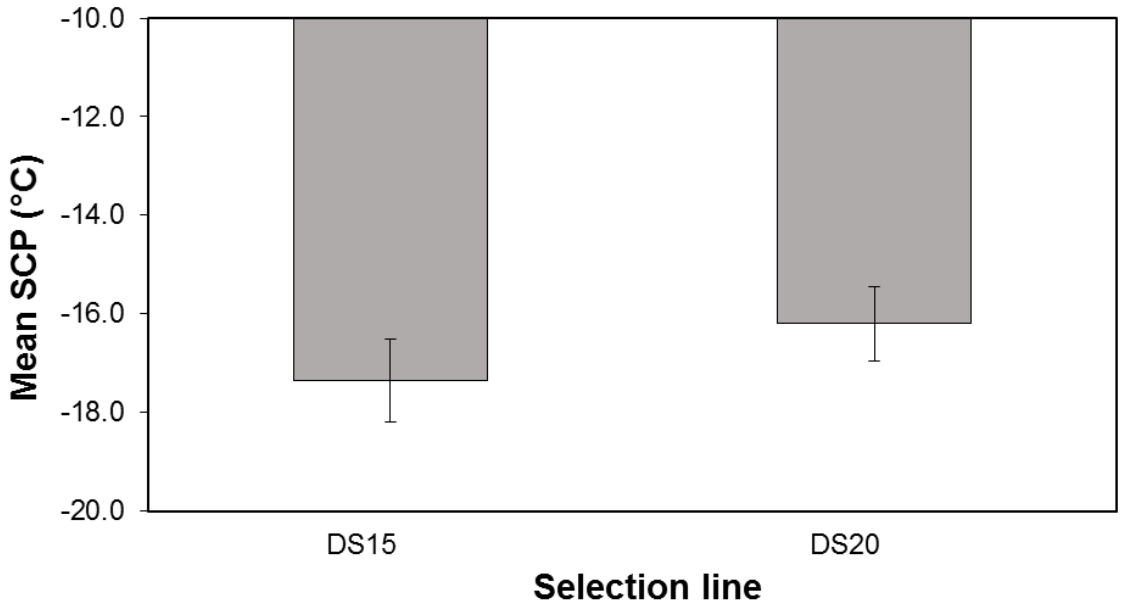


Figure 6.2 Mean SCP (± 1 S. E. M.) of *C. vicina* F1 generation L3 larvae selected for high larval incidence of diapause from adults acclimated to 15°C (DS15) or 20°C (DS20). Treatments were not significantly different from each other. *n* = 16 individuals for both treatments.

6.4.2.2 Lethal time

The tolerance of the F1 generation to chronic cold exposure, as determined by the time taken to induce LT_{10} and LT_{50} mortality at -8°C, varied significantly between DS and NDS lines but not between parental acclimation temperatures (Figure 6.3). This was determined through identification of non-overlapping 95% fiducial limits. There was no significant effect of adult acclimation temperature on cold tolerance, DS15 larvae exhibited the greatest LT50 and LT10 survival of any selection line.

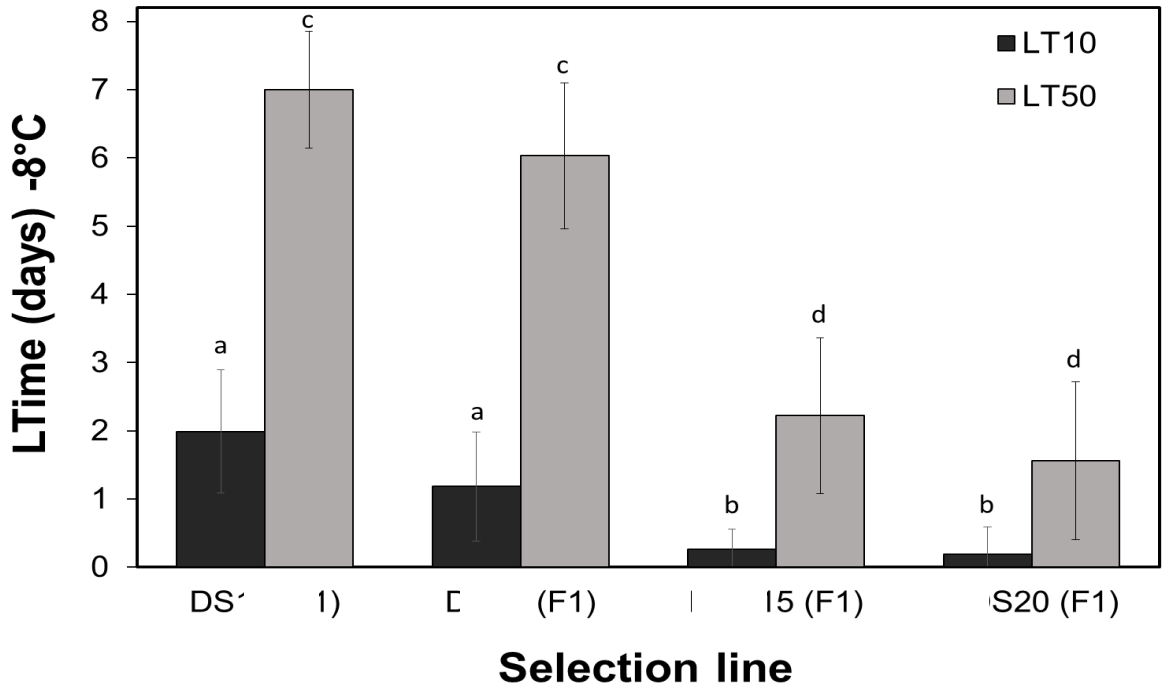


Figure 6.3 Mean Lethal Time (± 95% Fiducial limits) taken to induce 10% (LT10) and 50% (LT50) mortality following exposure to -8°C. Adults were cultured under a short-day photoperiod (LD 12:12 h) and acclimated to either 15°C (DS15 and NDS15) or 20°C (DS20 and NDS20) for 1 generation (F1). *n* = 480 individuals per treatment. Means associated with different letters are significantly different.

6.4.3 F2 generation, 20°C acclimated line

6.4.3.1 Supercooling capacity

There was a significant difference in the supercooling capacity of the F2 generation DS20 and NDS20 larvae ($F_{1,58}$ = 4.60; P<0.05) (Figure 6.4).

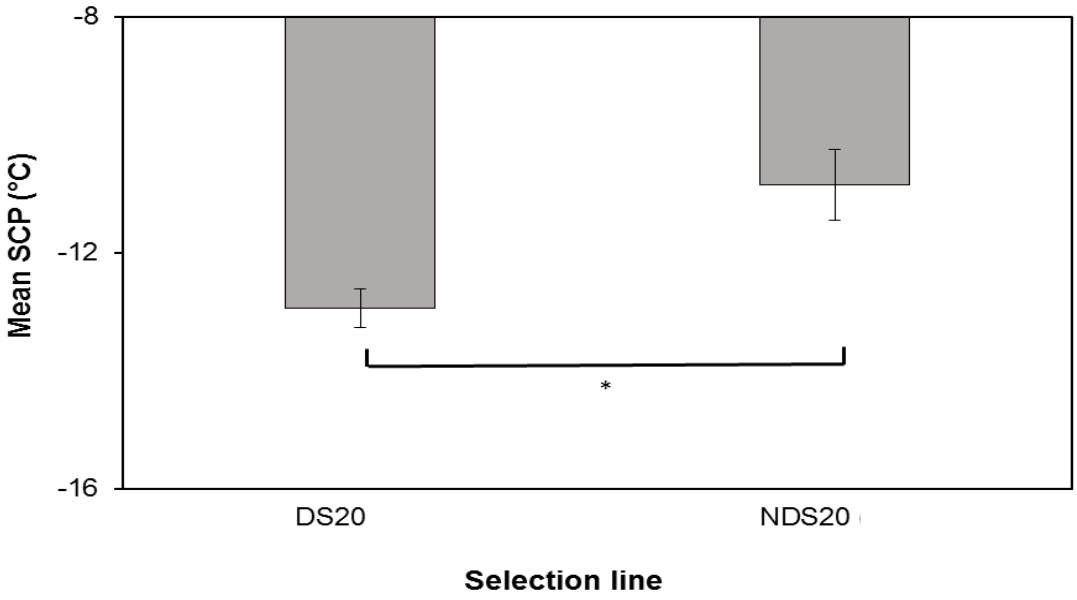


Figure 6.4 Mean SCP (± 1 S. E. M.) of the F2 generation L3 larvae selected for high (DS20) and low (NDS20) larval incidence of diapause from adults acclimated to 20°C. *n* = 30 individuals per treatment. * *P*<0.05 (Bonferroni *post-hoc*).

6.4.4 F5 generation, 15°C acclimated line

6.4.4.1 Supercooling capacity

There was a significant difference in the SCP of larvae ($F_{1,42}$ = 4.60; *P*<0.05) (Figure 6.5).

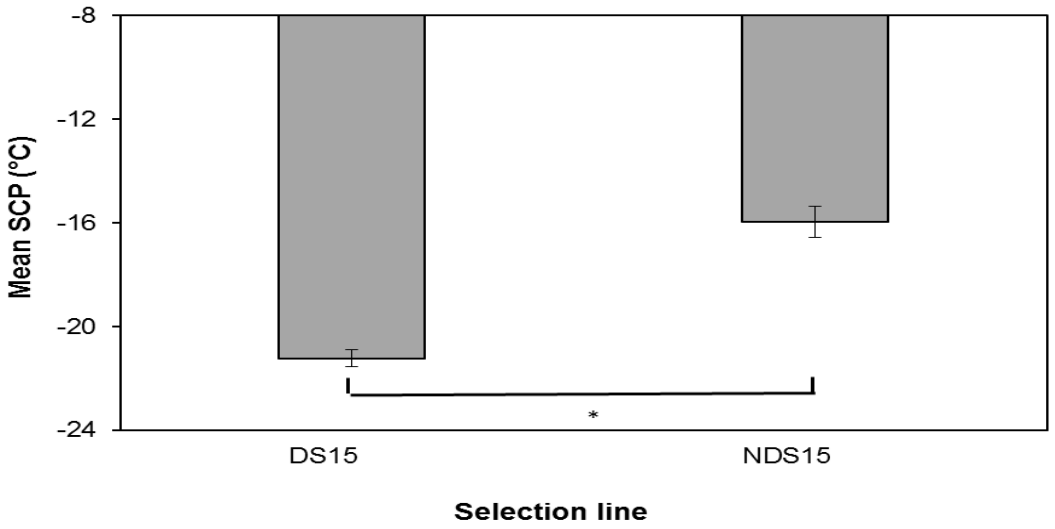


Figure 6.5 Mean SCP (± 1 S. E. M.) of the F5 generation L3 larvae selected for high (DS15) and low (NDS15) larval incidence of diapause from adults acclimated to 15°C. *n* = 44 individuals in total. * *P*<0.05 (Bonferroni *post-hoc*).

6.4.4.2 Lethal Time

The tolerance of chronic cold exposure for F5 generation DS15 and NDS15 larvae, as determined by the time taken to induce LT_{10} and LT_{50} mortality at -8°C, is presented in Figure 6.6.

The cold tolerance of DS15 larvae was significantly greater than DS20 larvae in the time taken to induce LT50 survival but not LT10 survival, as determined by non-overlapping fiducial limits.

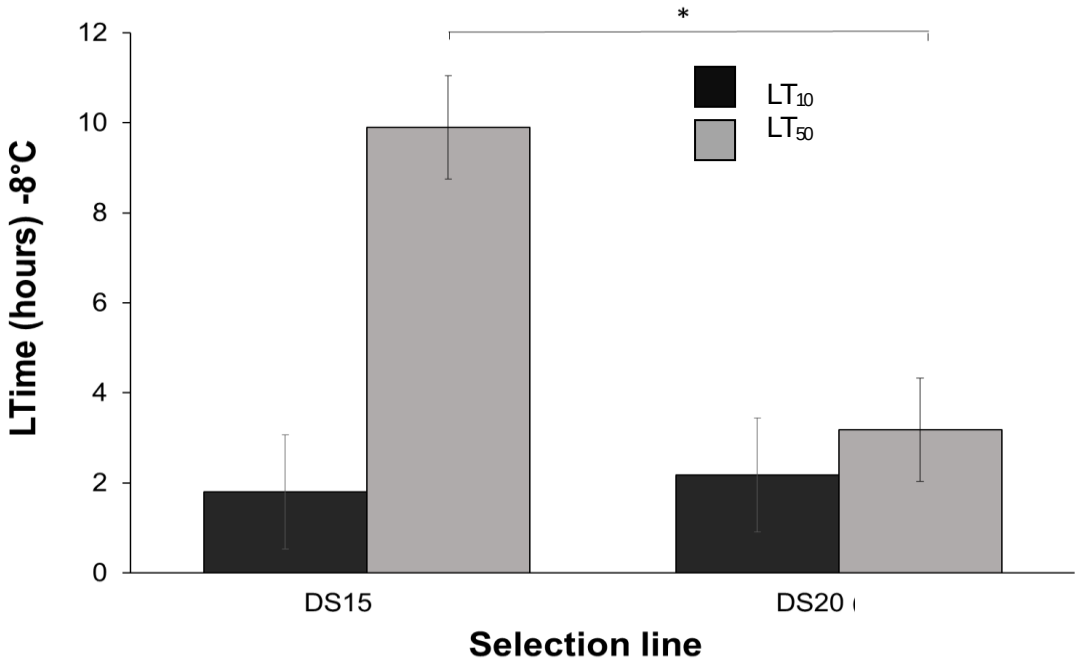


Figure 6.6 Mean Lethal Time (± 95% Fiducial limits) taken to induce 10% (LT10) and 50% (LT50) mortality following exposure to -8°C for the generation F5 larvae. Larvae were selected for a high (DS15) and low (NDS15) incidence of diapause from a parental population acclimated to 15°C. *n* = 480 individuals per treatment. * significantly different, determined by non-overlapping ± 95% Fiducial limits.

6.4.5 Realised Heritability

The response to selection for diapause incidence from generation F0 to F2 is presented in Table 6.1. Overall, there was a greater h^2 for low diapause incidence than high diapause incidence. The overall estimate of h^2 for diapause incidence is 0.54 ± 0.08.

The response to selection for supercoiling capacity from generation F0 to F2 is presented in Table 6.2. Overall there was a greater h^2 for low diapause incidence than high diapause incidence, although the difference was not as pronounced as for diapause incidence (Table 6.2). The overall estimate of h^2 for supercooling capacity is 0.59 ± 0.04.

Table 6.1 The realised heritability (h^2) (± 1 S.E.M.) for diapause (D) incidence in *C. vicina* larvae selected for high diapause incidence at 15 and 20°C (DS15 and DS20) and low diapause incidence at 15 and 20°C (NDS15 and NDS20). The overall estimate of

Selection line		Heritability (h^2) ± S.E.
High D incidence	DS15	0.08 ± 0.01
High D incidence	DS20	0.38 ± 0.04
Low D incidence	NDS15	0.67 ± 0.03
Low D incidence	NDS20	1.00 ± 0.23
Pooled high		0.23 ± 0.03
Pooled low		0.84 ± 0.13
Total pooled		**0.54 ± 0.08**

Table 6.2 The realised heritability (h^2) (± 1 S.E.M.) for supercoiling capacity in *C. vicina* larvae selected for high diapause (D) incidence at 15 and 20°C (DS15 and DS20) and low diapause incidence at 15 and 20°C (NDS15 and NDS20).

Selection line		Heritability (h^2) ± S.E.
High D incidence	DS15	0.70 ± 0.03
High D incidence	DS20	0.38 ± 0.05
Low D incidence	NDS15	0.83 ± 0.03
Low D incidence	NDS20	0.64 ± 0.02
Pooled high		0.54 ± 0.04
Pooled low		0.74 ± 0.03
Total		**0.59 ± 0.04**

6.5 Discussion

Selection lines, such as those presented in this chapter, have previously been used to investigate genetic differences associated with diapause in *S. bullata* (Henrich and Denlinger, 1982, 1983), in biocontrol studies on the predatory mites, *Amblyseius cucumeris* and *A. barkeri* (Houten et al., 1995), and in photoperiodism studies on *C. vicina* (Saunders and Cymborowski, 2003). Selection lines have also been used to determine heritability in the house mouse (*Mus musculus*) (Lynch, 1980; Swallow et al., 1998), *M. domesticus* (Gammie et al., 2006), the cricket (*Gryllus bimaculatus*) (Morrow and Gage, 2001) and *D. melanogaster* (Gilchrist and Huey, 1999).

This is the first time that selection lines have been used to determine whether cold tolerance is part of diapause, or a separate response programmed by the same photoperiodic and temperature cues, and thus only coinciding with diapause. This is also the first time that selection lines have been used to determine the heritability of diapause incidence and supercoiling capacity. This was achieved by establishing non-diapause selection lines, using the proportion of larvae that naturally avert diapause within a diapause-programmed population. Diapause and non-diapause selection lines were cultured under diapause-inducing conditions for multiple generations and subsequent changes in diapause incidence, supercooling capacity and chronic cold tolerance were investigated.

For the F2 and F3 generations, the incidences of larval diapause for both the diapause (DS15 and DS20) and non-diapause (NDS15 and NDS20) selection lines were lowest following selection under an adult acclimation temperature of 20°C rather than 15°C (Figure 6.1). This observation confirms that higher adult temperatures reduce the incidence of L3 larvae entering diapause, a relationship which has been identified in more northerly strains of *C. vicina* (51°N and 65°N) (McWatters and Saunders, 1998), in the mosquito, *Aedes togoi* (Kappus and Venard, 1967) and in the parasitic wasp, *Trichogramma evanescens* (Vinogradova and Zinovjeva, 1972). It has been suggested that faster development under

elevated temperatures results in the sensitive stage detecting a weaker-diapause inducing signal (Mousseau and Dingle, 1991; Saunders, 1997a). For example, maternal larvae and pupae of *A. atropaplus* must experience a minimum of nine short-day photoperiods or diapause is averted in eggs of the subsequent generation (Beach, 1978). Similarly, in *C. vicina* older adults produce a higher incidence of larval diapause, possibly due to the accumulation of additional photoperiod cues (Saunders, 1997a). Diapause incidence was greatest in the DS15 strain, with 100% of larvae entering diapause in the F5 generation, and lowest in NDS20, with all larvae averting diapause by the F1 generation.

The establishment of diapause (DS15 and DS20) and non-diapause (NDS15 and NDS20) selection lines was associated with changes in the supercooling capacity of L3 larvae (Figure 6.2, Figure 6.4 and Figure 6.5). In the F1 generation, the supercooling capacity was greater in DS15 than DS20 larvae (-17.3 ± 0.8°C and -16.2 ± 0.8°C, respectively) following acclimation at 5°C for three months, but these differences were not significant (Figure 6.2). These findings indicate that the cross generation effects of parental acclimation temperature, presented in Chapter 2 (section 2.3.3) are masked through the process of acclimation. In Chapter 2, diapause larvae from adults acclimated to 15°C were identified as having a greater supercooling capacity than larvae from adults acclimated to 20°C. Acclimation to non-severe temperatures has also been shown to heighten survival at more extreme temperatures for a number of species such as *Graphosoma lineatum*, *D. melanogaster* and *M. persicae* (Slachta et al., 2002; Kristensen et al., 2008; Alford et al., 2012) and was explored in detail, with respect to rapid cold hardening, in Chapter 3. These findings could be of importance as climate change continues, and parental populations are increasingly exposed to elevated late-autumn temperatures (Chapter 2). The response of parental adults experiencing higher autumn temperatures (Bale and Hayward, 2010), in reducing the supercooling capacity of diapause progeny, could be diminished through the process of acclimation. This could prove advantageous as larvae are still expected to encounter low temperatures within the soil layer. There were also differences in the supercooling capacity

of diapause and non-diapause selection lines. In the F2 generation, DS20 larvae displayed a significantly greater supercooling capacity than NDS20 larvae (Figure 6.4), while in the F5 generation, DS15 larvae exhibited a stronger supercooling capacity than NDS15 larvae (Figure 6.5). This confirms that heightened supercooling capacity is part of the diapause response, and is not expressed in response to photoperiodic and temperature cues experienced by non-diapause larvae. For the majority of species, supercooling capacity is not indicative of the lower limit of survival, with mortality often experienced at temperatures above the SCP (Bale, 1987; Renault et al., 2002). For this reason, the chronic cold tolerance of larvae following -8°C was also investigated (Figure 6.3 and Figure 6.6).The ability to withstand chronic cold exposure in the F1 generation was greater for the diapause than non-diapause selected lines (Figure 6.3). These differences were observed between DS20 and NDS20 larvae and between DS15 and NDS15 larvae. As was observed for supercooling capacity, these findings confirm that increased cold hardiness is part of the diapause response, and is not expressed in response to photoperiodic and temperature cues experienced by non-diapause larvae. Diapause is therefore fundamental in the expression of seasonal cold hardiness, irrespective of parental photoperiod exposure. This corroborates with the current understanding of diapause as pre-emptive response to periods of adverse environmental conditions (Denlinger, 2002; Koštál, 2006) and further substantiates the link between increased cold hardiness and diapause incidence (Schmidt et al., 2005; Schmidt and Paaby, 2008).Differences in cold hardiness between DS15 and NDS15 larvae in the F5 generation were less obvious than in the F1 generation. Both lines encountered substantial mortality following -8°C exposure, but DS15 larvae were still more cold tolerant than NDS15 larvae. It is unclear why acclimation to 15°C until the F5 generation reduced cold hardiness of both the diapause and non-diapause larvae, especially given associated increase in supercooling capacity. It is possible that the reduced cold tolerance is an artefact of the low genetic diversity which has arisen from inbreeding over multiple generations. Reduced cold hardiness is unlikely to be a natural response to consecutive generations entering diapause, as this response was also detected in the non-diapause line, and because at high latitudes

C. vicina is believed to be a univoltine species (Vinogradova, 1986; McWatters and Saunders, 1996).

Realised heritability provides an estimate of the additive genetic variation present within a population. As a general rule, traits closely associated to fitness display a low heritability, while traits more distantly related to fitness have higher heritability (Lynch, 1980; Mousseau and Roff, 1987; Swallow et al., 1998; Gilchrist and Huey, 1999; Morrow and Gage, 2001; Gammie et al., 2006). An overview of heritability estimates for numerous physiological and morphological characteristics in six invertebrate species is provided in Table 6.3.

Table 6.3 Heritability estimates for a variety of physiological and morphological characteristics in six invertebrate species. Data taken from Mousseau and Roff (1987) and Gilchrist and Huey (1999).

Species	**Characteristic**	**Heritability (h^2)**
Apis mellifera	Chill coma	0.15
	0^2 consumption	0.13
Eurytemora affinis	Temperature tolerance	0.76
Eurytemora herdmani	Body length	0.54
Gryllus fimus	Wing length	0.40
Tribolium castaneum	Fecundity	0.30
Drosophila melanogaster	Upper knockdown temperature	0.02 – 0.15
	Lower knockdown temperature	0.00 – 0.30

The rate of increase in fitness of any organism at any time is equal to its genetic variance in fitness at that time (Fisher, 1930; Mousseau and Roff, 1987). The realised heritability for a high incidence of larvae diapause (h^2 = 0.03; Table 6.1) is low when compared to characteristics that have been selected for in other invertebrate species (Table 6.3). The low heritability suggests that a high incidence of larval diapause is closely associated to fitness. These findings are in accordance with the understanding of diapause as a pre-requisite of winter survival for the majority of temperate insects (Bale and Hayward, 2010). It is advantageous to have a low heritability in the diapause phenotype, as this ensures that a large proportion of the population will enter diapause over the winter period, regardless of

external pressures. This low heritability could prove advantageous under future climatic conditions, with a predicted increase in average autumn temperatures (IPCC, 2013). Increased autumn temperatures are still expected to be followed by periods of low winter temperature. A low heritability in the diapause phenotype will result in a large proportion of larvae continuing to enter diapause, and therefore having cold tolerance mechanisms in place, facilitating winter survival.

As opposed to increased diapause incidence, there was a high degree of heritability associated with low diapause incidence (h^2 = 0.84; Table 6.1). This indicates that there is significant genetic variation responsible for the proportion of the population able to abort diapause. It is likely that this bet-hedging strategy has evolved in order to allow a proportion of the population to take advantage of warmer winters, through continued development. It is easy to perceive that over winter periods with low ambient temperatures, there will be significant mortality in the population aborting diapause. This selective pressure will ensure that the diapause phenotype will remain dominant within natural populations.

The realised heritability for supercoiling capacity (h^2 – 0.54 and 0.74 for high and low diapause selection lines, respectively; Table 6.2) is relatively high compared to characteristics measured in other invertebrates (Table 6.3). This suggests that supercoiling capacity is not closely related to fitness. This can be explained by the fact that the supercoiling capacity of both diapause and non-diapause larvae (-18.9˚C and -12.3˚C; see Chapter 2, section 2.4.1) far exceeds that of the lowest temperatures experienced in the soil layer over the winter period (-4.7˚C between October 2009 and April 2013, Chapter 5). These findings suggest that cold tolerance may not be the primary selective pressure driving diapause in *C. vicina*. It is possible that other factors, such as reduced metabolic activity, delayed metamorphosis and the synchronisation of development with resource availability may have played a more pivotal role in the evolution of insect diapause.

6.6 Conclusions

For the majority of insects it is unclear whether diapause and cold hardiness are induced as part of a ubiquitous overwintering response or if they are induced independently, albeit over a similar timeframe, following detection of similar environmental stimuli, such as shortening photoperiods and declining temperatures (Denlinger, 1991; Pullin, 1996; Somme, 1999; Hodkova and Hodek, 2004). The current chapter identified a close association between diapause incidence, supercooling capacity and cold hardiness. Diapause initiation is believed to be a prerequisite of cold-hardiness for a number of species such as *S. crassipalpis*, Aulacorpha nigripennis and *Gratiana graminea* (Mansingh, 1974; Lee and Denlinger, 1985; Pullin and Wold, 1993; Watanabe and Tanaka, 1998; Manrique et al., 2012; Murata et al., 2013), but this assumption has until now been based on limited experimental evidence (Hodkova and Hodek, 2004).

The effects of environmental stimuli were disentangled from the diapause response through selection for diapause and non-diapause strains, under photoperiodic and temperature conditions known to induce diapause and enhance cold hardiness (Saunders and Hayward, 1998). Reduced cold tolerance associated with the non-diapause selected line strongly suggests that diapause is a pre-requisite for implementing seasonal increases in cold hardiness.

The low heritability in diapause incidence suggests that this phenotype is closely associated to fitness. However, the high heritability in supercoiling capacity indicates that cold tolerance may not be the primary factor that has driven the evolution of insect diapause. It is possible that other phenotypes, such as delayed development and reduced resource requirements are the primary factors in the evolution of diapause. The findings presented here also further substantiate the cross generation response to parental acclimation temperature presented in Chapter 2. The culturing of diapause larvae over multiple generations at the lower adult acclimation temperature (15°C) further enhanced the supercooling capacity of diapause

larvae. The mean SCP of first generation diapause larvae from 15°C adults was identified as -18.9°C (Figure 2.2). In the F5 generation, diapause larvae from parents acclimated to 15°C exhibited a mean SCP of -21.2°C. The opposite was observed in diapause larvae from 20°C-acclimated adults, with the mean SCP increasing from -15.3°C in the first generation to -12.9°C by the F2 generation. The same trend was also observed for non-diapause larvae, with adults acclimated to 15°C producing first generation larvae with a mean SCP of -11.4°C (Figure 2.2) and F5 generation non-diapause larvae with a mean SCP of -15.9°C. These findings indicate that while there is cross generational effect of temperature acting on non-diapause larvae over one generation (Chapter 2), an effect is observed following parental acclimation over multiple generations. While a similar response was also observed for chronic cold tolerance following -8°C exposure, this relationship was less obvious due to the effects of inbreeding and reduced cold hardiness expressed by the F5 generation selection lines (Figure 6.6).

The relationship between environmental stimuli, diapause and cold hardiness could be further investigated through the established of diapause and non-diapause selection lines under photoperiodic and temperature conditions known not to induce diapause. It is now known whether the proportion of non-diapause-programmed larvae that dislay pupariation also display an increased cold hardiness phenotype. Such an investigation would provide an insight into whether some individuals are pre-determined to enter diapause, irrespective of prevailing environmental conditions. The relationship between diapause and cold hardiness is further investigated in the following chapter, with a particular focus on the role of amino acids during diapause.

CHAPTER 7

THE EFFECTS OF DIETARY SUPPLEMENTATION WITH AMINO ACIDS ON DEVELOPMENT, COLD TOLERANCE AND METABOLISM

7.1 Abstract

The diapause phenotype in the blow fly, *Calliphora vicina*, is associated with delayed development as third-instar larvae, an increased supercooling capacity and heightened tolerance to acute and chronic cold exposure. A metabolomic analysis of *C. vicina* identified that diapause larvae up-regulate the amino acid, alanine. The upregulation of alanine is often indicative of conversion to glycolysis, but could also be a direct response to preceding cold, with a possible role in cryopreservation.

In this chapter, non-diapause larvae were provided with a dietary source of alanine. Alanine-supplemented larvae displayed delayed development and lower mean SCPs (by 3.3°C) than control non-diapause larvae. Also, survival following 2 h exposure to -0°C increased by 31.7%, and the time taken to induce 50% mortality at -4°C was increased by 4.2 d. While development time and SCPs remained below that of diapause larvae, acute and cold tolerance exceeded that of diapause larvae. Additionally, dietary alanine was associated with a moderate increase in the cold tolerance of diapause larvae. Supplemented larvae developed through to adults exhibited a decrease in chill coma of 2°C when compared to control adults.

A metabolomics comparison of non-diapause and non-diapause alanine supplemented larvae failed to identify an accumulation of alanine in supplemented larvae. However, supplemented larvae up-regulated succinate, glycine and tyramide. Although the exact role of alanine is not identified in this chapter, it does provide the first evidence that dietary supplementation with alanine has a clear effect on the expression of diapause-associated phenotypes.

7.2 Introduction

Metabolomics is a powerful comparative tool in molecular biology. It provides a holistic snap-shot of the small molecules found in the tissues and fluids of an organism, including sugars, amino acids, metabolic intermediates and polyols (Hawes et al., 2008). To date there have been six metabolomic studies on insect diapause. These have focused on the flesh fly, *S. crassipalpis* (Michaud and Denlinger, 2007), the drosophilid, *Chymomyza costata* (Kostál et al., 2011), the aphid parasitoid, *Praon volucre* (Colinet et al., 2012), the cotton bollworm, *Helicoverpa armigera* (Zhang et al., 2012; Xu et al., 2012) and the blow fly, *Calliphora vicina* (Johnson, 2013). The first of these studies, performed on *S. crassipalpis*, identified 64 metabolites in diapause and non-diapause pupae. Five of these metabolites were significantly up-regulated during diapause and eight were down-regulated. The majority of down-regulated metabolites were intermediate compounds of the Krebs cycle (e.g. fumarate and citrate), the process of aerobic energy production. The majority of up-regulated metabolites were associated with glycolysis (such as glycerol, glucose, alanine and pyruvate), the process of anaerobic energy production (Michaud and Denlinger, 2007). It has been suggested that a reduction in the Krebs cycle prevents the accumulation of the toxic end-product, lactic acid (Michaud and Denlinger, 2007). In contrast, glycolysis can continue in anaerobic conditions and is associated with the accumulation of non-toxic compounds, such as alanine (as first observed in sea grass; see Touchette and Burkholder, 2000). A decrease in Krebs cycle intermediaries has also been identified in *P. volucre* (Colinet et al., 2012), *H. armigera* (Xu et al., 2012) and *C. vicina* (Johnson, 2013). Metabolomic studies, in addition to identifying compounds associated with energy production, have also detected metabolites with a possible role in cold tolerance (also termed cryoprotection).

Cryoprotectants, or cold responsive metabolites, are typically sugars and polyols such as glycerol and glucose that accumulate to high levels in response to low temperature (Storey, 1997; Marshall and Sinclair, 2010). Such compounds display a colligative effect with other

solutes and depress the internal melting and freezing point, thereby reducing the risk of freezing, and thus enhancing the chances of overwinter survival (Michaud and Denlinger, 2007; Colinet et al., 2012; Johnson, 2013).

A number of metabolomic studies, in addition to identifying the up-regulation of sugars and polyols, have also identified substantial change in the regulation of free amino acids (Koštál et al., 2009; Colinet et al., 2012). For example, diapause in *S. crassipalpis* was associated with a nine-fold increase in alanine (Michaud and Denlinger, 2007). The authors of the study proposed that alanine accumulation was a likely response to glycolysis, although did not dismiss a possible role in heightening cold tolerance. Alanine has been associated with enhanced cold hardiness in previous studies on the gall fly, *Eurosta solidaginis* (Churchill and Storey, 1989) *Belgica antarctica* (Michaud et al., 2008), and the oriental corn borer, *Ostrinia furnacalis* (Goto et al., 2001). However, alanine accumulation in response to seasonal low temperatures in the fall webworm, *Hyphantria cunea*, was not associated with an increase in cold tolerance (Li et al., 2001). Thus, the role of alanine in insect diapause remains unclear. It is possible that alanine is up-regulated to assist in cold protection, or alternatively that it accumulates as a by-product of other physiological adaptations. There is some evidence that alanine may contribute towards cold tolerance. For example, *O. furnacalis* (Goto et al., 2001), removed from the wild during periods of low temperature, display increased levels of alanine and heightened cold tolerance, although a direct relationship was not confirmed. Alanine is believed to be responsible for an increased supercooling capacity in cold tolerant life stages of the codling moth, *Cydia pomonella* (Rozsypal et al., 2013). Alanine may help to stabilise proteins at low temperatures (Carpenter and Crowe, 1988), thereby reducing the potential of low temperature mortality. Its colligative properties are similar to those of glucose, which would also make it a useful compound in preventing ice formation at low temperatures (Michaud and Denlinger, 2007).

Direct evidence of amino acids contributing towards cold tolerance comes from studies on proline. Proline has been identified as the dominant metabolite in diapausing *C. costata* and

in cold-acclimated *D. melanogaster* (Koštál et al., 2011a; Kostál et al., 2011b). *D. melanogaster* is a freeze-avoiding species that experiences complete mortality following direct exposure to -5°C (Koštál et al., 2011a). However, larvae can tolerate 50% of their body tissues being frozen at -5°C if proline content is artificially increased (Koštál et al., 2012). Similarly, survival of *C. costata* non-diapause larvae following liquid nitrogen exposure increased from 0% to 36% when proline content was artificially increased (Kostál et al., 2011c). The above studies did not identify the exact mechanisms underpinning how proline contributed towards increased cold tolerance in *C. costata* or *D. melanogaster*, and it is not known whether proline is associated with cold hardiness in other species. It has been suggested that proline may help to stabilise proteins (Rozsypal et al., 2013), alleviate stress experienced by the phospholipid membrane (Koštál et al., 2012) and its accumulation in exogenous spaces reduces the osmotic pressure experienced by cells, thereby preventing cell shrinkage (Kostál et al., 2011b). This chapter aims to further our understanding of the role of amino acids in cold protection, with particular focus on alanine and proline.

C. vicina represents a particularly good model to investigate the potential role of alanine and proline in diapause and cold tolerance. Recent work from our laboratory found both of these to be up-regulated within the diapause programme (Johnson, 2013). Alanine was the dominant amino acid in diapausing *C. vicina*, with a 2.56-fold increase compared to non-diapause larvae, while proline displayed a 1.12-fold increase (Johnson, 2013). The metabolomic comparison of diapause and non-diapause *C. vicina* larvae is shown in Figure 7.1.

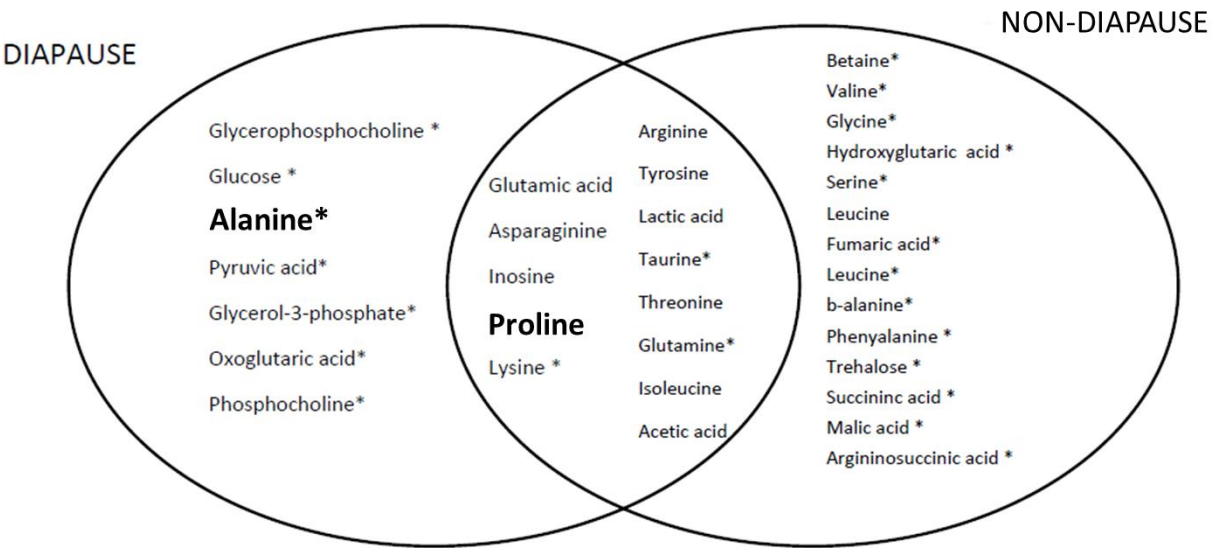


Figure 7.1 A Venn diagram of the metabolites identified in diapause and non-diapause third-instar *C. vicina* larvae. Metabolites in separate circles were significantly different between conditions at $P<0.001$ (using an ANOVA) while metabolites in the inner overlapping circle were not significant. * designates that the metabolite was only detected in one condition. Based on results from Johnson (2013).

This chapter aims to build on this metabolomic study of *C. vicina* to investigate whether the amino acids, alanine and proline, contribute directly towards an increased cold hardiness associated with the diapause state. In addition to having a role in cold protection during diapause, it is also possible that these amino acids could increase cold tolerance in life stages outside of diapause. The molecular changes underpinning diapause have been linked to cold tolerance mechanisms associated with other cold tolerance phenotypes. For example, rapid cold hardening (an immediate response to sudden environmental fluctuations) and diapause are both associated with the synthesis of low molecular weight polyols and amino acids (Koštál and Simek, 2000; Overgaard et al., 2007), the up-regulation of HSPs (Rinehart et al., 2007), and a lowering of the SCP (Bale et al., 1984). It is therefore possible that amino acids could play a ubiquitous role in cold tolerance. This is not currently substantiated by experimental evidence. To investigate this further, the effect of providing individuals with a dietary source of alanine and proline was investigated in relation to cold tolerance in both the larval and adult life stage.

For the larval life stage, it was investigated whether the dietary intake of alanine or proline in non-diapause larvae contributed towards the expression of diapause-specific phenotypes. The phenotypes measured were delayed pupariation (Figure 5.3), enhanced supercooling capacity (Figure 2.2) or increased cold hardiness (Figure 2.4). Increased cold tolerance in dietary enriched non-diapause larvae would be a good indication of amino acids contributing towards low temperature survival within the diapause state. It was also investigated whether the provision of dietary alanine has the ability to enhance the supercooling capacity or cold hardiness of the diapause life stage, which would again provide good evidence for amino acids playing a role in cold protection. While previous studies on species such as *C. costata* and *D. melanogaster* have enhanced cold tolerance through the provision of dietary amino acids (Koštál et al., 2011a), they have not addressed how proline influenced other metabolic pathways. This chapter seeks to identify how the dietary intake of alanine affects other metabolic pathways. This was achieved by performing a metabolomic comparison between diapause and non-diapause supplemented larvae. The metabolomic comparison will also provide an indication of the quantity of alanine accumulated within supplemented larvae.

Finally, the effect of dietary alanine on the cold tolerance of adult *C. vicina* was also investigated. Using adults, it was determined whether (1) larvae provided with a dietary source of alanine were more cold tolerant in the adult life stage and (2) whether adults provided with a direct source of alanine were more cold tolerant than their non-supplemented counterparts. This was investigated based on adult supercooling capacity and thermal activity thresholds (as investigated in Chapter 4). An association between increased cold tolerance and dietary alanine in the adult life stage would also indicate that amino acids play a role in cold protection over multiple life stages.

7.3 Materials and methods

7.3.1 Experimental cultures

The establishment of experimental cultures followed that outlined in Chapter 2, section 2.3.2, the only exception being that cow mince, rather than pig liver, was provided as the protein source on d 4, 6, and 8 and every day thereafter. Incorporating amino acids into mince for dietary manipulation was much easier than their incorporation into liver. Controls confirmed that maturing larvae on mince instead of liver had no effect on the cold tolerance phenotype or on development.

Adult cultures were held at 15°C under a longday photoperiod (LD 18:6 h), for the production of non-diapause larvae, and under a shortday photoperiod (LD 12:12 h) for the production of diapause larvae. Diapause and non-diapause larvae are morphologically indistinguishable, but, diapause larvae can be identified as larvae not pupariating by d 30 post-oviposition (Richard and Saunders, 1987).

7.3.2 Larvae

7.3.2.1 Dietary manipulation treatments

Mince was provided as a source of protein and site of oviposition on d 4, 6, 8 and every day thereafter. From d 12 onwards, mince was removed from adult cultures and the oviposited eggs held under high humidity in complete darkness for 24 h to encourage hatching. Following hatch, approximately 200 larvae were transferred to 20 g of ‘amino acid-augmented’ mince for development until the time of mass wandering, at which time feeding ceased and larvae wandered from the food source to the underlying sawdust. The mince provided for larval feeding was augmented with either alanine or proline using the following concentrations:

- 0 g alanine or proline: 100 g mince for diapause (D) and non-diapause (ND) larvae (D-Ala0 and ND-Ala0; D-ProO and ND-Pro0) (Control group)
- 1 g alanine or proline: 100 g mince (D-Ala1 and ND-Ala1; D-Pro1 and ND-Pro1)
- 5 g alanine or proline: 100 g mince (D-Ala5 and ND-Ala5; D-Pro5 and ND-Pro5)
- 10 g alanine or proline: 100 g mince (D-Ala10 and ND-Ala10; D-Pro10 and ND-Pro10).

Non-diapause larvae were used on d 15 post-oviposition and diapause larvae on d-30 post-oviposition. At this time diapause and non-diapause larvae are considered to be at the same stage of development.

7.3.2.2 Survival assessments

The effect of alanine and proline augmentation on mortality in the absence of any stress treatment was determined for all larval treatments. Mortality associated with larval dietary manipulation was determined by placing larvae in a Petri dish containing a layer of sawdust following mass wandering. Larvae continued to be held in complete darkness at 11°C and survival was determined as successful adult eclosion. Six replicates of 10 individuals were performed for each treatment.

The effect of amino acid augmentation on larval feeding stages prior to mass wandering was determined by assessing the proportion of living to dead larvae at the time of mass wandering.

7.3.2.3 Larval development time

The effect of amino acid augmentation on development was determined for ND-Ala1, ND-Ala5, ND-Ala10, and compared with ND-Ala0 and D-Ala0 larvae as untreated controls. Time to mass wandering represented the number of days post-oviposition taken for the larvae to cease feeding and relocate to the underlying sawdust. This was repeated six times with replicates of approximately 300 larvae.

Following mass wandering, groups of 10 third-instar larvae were transferred to Petri dishes containing a thin layer of sawdust, held in complete darkness at 11°C and the time taken for 50% of individuals to pupariate and then eclose as adults was recorded. This was repeated six times.

7.3.2.4 Supercooling capacity

Supercooling capacity was determined through identification of individual SCPs as described in Chapter 2, section 2.3.3. This was performed for all non-diapause and diapause larvae, with *n* = minimum of 15 larvae per treatment.

7.3.2.5 Acute cold tolerance

Tolerance to acute cold exposure (discriminating treatment) was determined for non-diapause larvae as survival following 2 h exposure to -10°C. This temperature was determined as inducing 80% mortality for non-diapause larvae in Chapter 3, Section 3.4.1. Individuals were placed in 50 ml test tubes and plunged into an alcohol bath (Grant LTD D6C, Grant Instruments, Cambridge, UK) pre-set at -10°C. Survival was assessed as successful development to the adult life stage for larvae held at 11°C in complete darkness, with *n* = 6 × 10 replicates per treatment. A control group received the same treatment although instead of being exposed to -10°C it continued to be held at 11°C.

7.3.2.6 Chronic cold tolerance

The time taken to induce 10% (LT10) and 50% (LT50) mortality at -4°C and -8°C was determined for all larval non-diapause and diapause alanine treatments following the procedure described in Chapter 2, section 2.3.4. Briefly, three groups of 10 third-instar larvae were placed in 10 ml glass vials containing 1 cm of sawdust, held in a programmable incubator at either -4°C or -8°C, and removed at 3 d intervals for the first 18 d and then 3 d or 6 d intervals until 50 d. Survival was assessed as successful adult eclosion at 11°C, with *n* = minimum of 180 individuals per treatment.

7.3.3 Adults

7.3.3.1 Dietary manipulation treatments

A proportion of ND-Ala10 larvae was developed through to the adult life stage by holding larvae and pupae in complete darkness at 11°C until the time of adult eclosion. Eclosed adults were provided with water and sugar, held under a long-day photoperiod (LD 18:6 h) at 15°C and used for experimentation at d 6 post-eclosion. This was to investigate the effect of amino acids provided during the larval life stage to cold tolerance expressed in adults of the same generation.

The effect of alanine was also investigated by providing stock culture (Chapter 2, section 2.3.1) newly eclosed adults with a diet augmented with amino acids. Newly eclosed adults were established under a long-day photoperiod (LD 18:6 h) and provided with *ad libitum* access to water and sugar. Dietary alanine was provided in the following amounts dissolved in 100 ml of water source under the following concentrations; 0 g, 1 g, 5 g and 10 g.

Adults were then ready for experimental use on d 6 post-eclosion.

7.3.3.2 Survival

The effect of alanine substitution on survival was investigated for all treatments. The number of dead individuals was noted on d 6 after the culture had been established. This was repeated five times with approximately 100 mixed sex adults per culture.

Survival in adults provided with alanine during the larval stage was assessed as successful adult eclosion for all treated larvae.

7.3.3.3 Supercooling capacity

Supercooling capacity was determined through identification of individual SCPs as described in Chapter 2, section 2.3.3, for all adult treatments (*n* = minimum of eight adults per treatment). The low adult sample size was due to the high levels of adult mortality following the provision of dietary alanine.

7.3.3.4 CT_{min} and chill coma

CT_{min} and chill coma were identified following gradual cooling at 0.5°C min^{-1} as described in Chapter 3, section 3.3.6. This was performed for all adult treatments, (*n* = minimum of eight per treatment).

7.3.4 Metabolomic characterisation

7.3.4.1 Metabolomic extraction and fingerprinting

Frozen larvae (ND-Ala0 [d 15 post oviposition], ND-Ala5 [d 15 post oviposition] and D-Ala0 [d 30 post oviposition]) were held on dry ice, weighed and then transferred to homogenisation tubes. An ice cold methanol:chloroform:water (2:1:8) mixture was added to each sample. Larvae were then homogenised using a Precellys-24 bead-based homogenizer (Stretton Scientific Ltd, Stretton, UK). Homogenised larvae were transferred to Eppendorf tubes (Eppendorf Ltd, USA), vortexed for two minutes and then centrifuged (20s vortexing for 20 min). This process resulted in a phase separation of an upper (polar) sample and a lower (non-polar) sample. For the metabolomic analysis only the polar extract was required (although the non-polar extract was frozen at -80°C in case it was required for lipidomic analysis at a later time). For the polar extract, 130 µl of sample was removed, dried for one hour and then transferred to -80°C until it was required for subsequent analysis. Eight larval replicates were used for each treatment.

The running of samples had to be performed by members of the NERC Nuclear Magnetic Resonance (NMR) facility at the University of Birmingham, UK. Before analysis, samples were re-suspended in 650 µl of sodium phosphate buffer solution. Samples were then centrifuged for 10 min at 21000 g to ensure complete dissolution and 600 ml of each sample transferred to 5 mm NMR tubes (Norell Inc, Landisville, Pennsylvania, USA)

The particle separation of samples was performed using a Bruker Avance 500 MHz NMR spectrometer equipped with a 5 mm cryoprobe (Bruker Biospin, Coventry, UK). The injection of samples was completely randomized and each sample was allowed to equilibrate for 5

min to the desired temperature (298 K). Samples were analysed on a DRX-500 NMR spectrometer (Bruker Biospin) operated at 500.18 MHz.

The NMR spectra were processed to a suitable format for analysis using the ProMetab software, running within MATLAB (Version 7.0, Mathworks, Cambridge, UK). This allowed analysis to be performed on the different spectral 'peak' abundances of metabolites associated with the different treatments.

7.3.5 Statistical analysis

Larval and adult survival, larval development time, supercooling points and acute cold tolerance data analysis was undertaken using SPSS (v. 20.0, IBM, New York, USA). Data were analysed using GLMs. Significant differences between groups were identified using the Bonferroni *post-hoc* test with an alpha threshold of $P<0.05$.

Larval chronic cold tolerance (LT) data were analysed using the statistical package Minitab 15 (Minitab, Coventry, UK) as described in Chapter 2, section 2.3.7.

Adult lower thermal activity thresholds (CT_{min} and chill coma) were analysed using separate GLMs as described in Chapter 3, section 3.3.8.

Metabolite identification was performed using a principal components analysis (PCA) of the NMR data for ND-Ala0, ND-Ala5 and D-Ala0 treatments. The PCA was produced using PLS_Toolbox (Version 3.5, Eigenvector Research, Manson, WA, USA) within MATLAB. The identity of each metabolic peak was performed using a fingerprint library which had been generated by the University of Birmingham NERC Facility. Potential metabolite matches were identified and conformation of identity was conducted using TopSpin in MATLAB.

Significant differences in individual metabolites were identified using an ANOVA in R (R Development Core Team), with $P>0.0001$ to prevent the likelihood of false positives being identified in the large dataset.

7.4 Results

7.4.1 Larvae

7.4.1.1 Survival

No difference in survival was noted between control and alanine-supplemented larval groups prior to mass wandering, with 100% survival for all treatments. Alanine augmented mince also had no effect on survival for non-diapause or diapause larvae between mass wandering and adult eclosion ($F_{3,236}$ = 0.85, P=0.55), with mortality not exceeding 1.6% for any treatment group (Table 7.1).

Feeding larvae on proline-augmented mince resulted in some reduced survival prior to mass wandering, and significant reductions in survival post-wandering for both non-diapause ($F_{3,236}$ = 22.16, P<0.001) and diapause ($F_{3,236}$ = 102.03, P<0.001) larvae, with very few larvae surviving the Pro10 treatment group (Table 7.1). Due to the high levels of mortality, it was not possible to produce sufficiently high numbers of individuals for subsequent analysis. Consequently, only the role of alanine supplementation on larval cold tolerance could be investigated.

Table 7.1 The effect of alanine and proline dietary augmentation on mean survival (± 1 S. E. M.) to adult eclosion for non-diapause and diapause *C. vicina* larvae. Larvae were developed on: 0 g amino acid: 100 g mince (Ala0 and Pro0, control group); 1 g: 100 g (Ala1 and Pro 1); 5 g: 100 g (Ala5 and Pro 5) or 10 g: 100 g (Ala10 and Pro 10). n = 6 × 10 individuals per treatment.

Amino acid conc. (g of amino acid: 100 g mince)	Ala0 or Pro0	Ala1 or Pro1	Ala5 or Pro5	Ala10 or Pro10
Non-diapause alanine survival (%)	100.0 ± 0	100.0 ± 0	98.0 ± 1.7	100.0 ± 0
Non-diapause proline survival (%)	100.0 ± 0	73.3 ± 7.6	3.8 ± 14.0	1.0 ± 5.2
Diapause alanine survival (%)	100.0 ± 0	100.0 ± 0	100.0 ± 0	98.0 ± 1.7
Diapause proline survival (%)	100.0 ± 0	83.3 ± 6.7	2.5 ± 6.2	0.2 ± 1.7

7.4.1.2 Development time

Dietary alanine had a clear effect on the time taken for *C. vicina* non-diapause larvae to develop from the egg to the adult life stage (Figure 7.2). There were significant differences in the time from egg oviposition to mass wander ($F_{3,24}$ = 6.70, P<0.001), mass wandering to pupariation ($F_{3,24}$ = 121.55, P<0.001) and from pupariation to adult eclosion ($F_{3,24}$ = 78.70, P<0.001) between treatment groups. *Post-hoc* analysis identified these to be significant differences between ND-Ala0 and ND-Ala10 for each developmental interval (P<0.05 for development time to mass wandering and pupariation and P<0.001 to adult eclosion), and between ND-Ala0 and ND-Ala1 in the time between mas wandering and pupariation (P<0.05) and mass pupariation and eclosion (P<0.05).

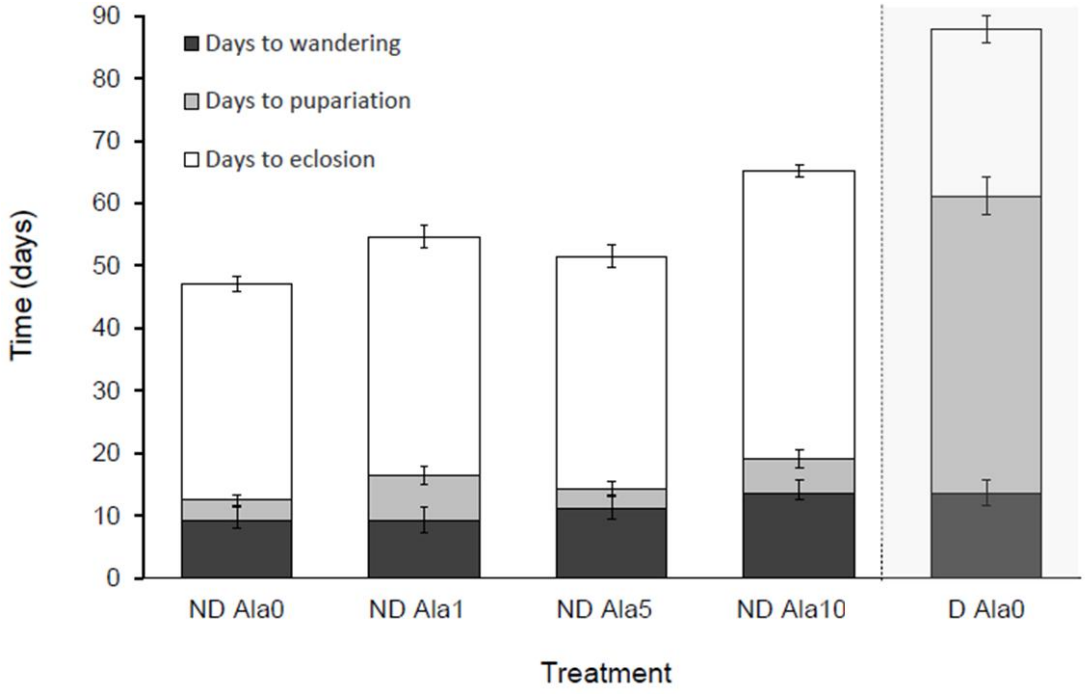


Figure 7.2 The effect of dietary alanine on the cumulative development time of non-diapause *C. vicina* larvae from the time of oviposition to mass wandering, pupariation and adult eclosion. Larvae were developed on 0 g (ND-Ala0), 1 g (ND-Ala1), 5 g (ND-Ala5) or 10 g (ND-Ala10) of alanine per 100 g mince. Diapause (D-Ala0) development time is provided for comparison. Bars show means ± 1 S. E. M. n = 6 × 10 individuals for each treatment.

7.4.1.3 Supercooling capacity

Dietary alanine had a significant effect on the SCPs of non-diapause larvae ($F_{3,69}$ = 4.65, *P*<0.005). In general, increased alanine intake was associated with an enhanced supercooling capacity. *Post-hoc* analysis determined these differences to be significant between ND-Ala0 and ND-Ala10 larvae (*P*<0.05) (Table 7.2).

Dietary alanine also had significant effect on the SCPs of diapause larvae ($F_{3,108}$ = 4.64, *P*<0.005). *Post-hoc* analysis determined these differences to be significant between D-Ala0 and D-Ala10 only (*P*<0.01).

Table 7.2 The effect of dietary alanine on the mean supercooling points (± 1 S. E. M.) and range for *C. vicina* non-diapause (ND) and diapause (D) progeny developed on 0 g (ND/D-Ala0), 1 g (ND/D-Ala1), 5 g (ND/D-Ala55) or 10 g (ND/D-Ala10) of alanine per 100 g beef mince. Means followed by different letters and in bold are significantly different from controls of the same group (D or ND) based on GLM *post-hoc* Bonferroni (*P*<0.05).

Treatment group	N	Mean ± SE (°C)	Range (°C)
ND-Ala0 (Control)	24	-10.3 ± 0.7^{a}	-18.7 to -5.9
ND-Ala1	15	-10.1 ± 0.6^{a}	-14.8 to -6.4
ND-Ala5	15	-13.2 ± 0.9^{a,b}	-17.9 to -7.5
ND-Ala10	15	**-13.6 ± 1.0^{b}**	-18.9 to -6.6
D-Ala0 (Control)	62	**-18.9 ± 0.5^{c}**	-24.3 to -8.5
D-Ala1	15	**-19.5 ± 0.7^{c}**	-24.3 to -15.1
D-Ala5	15	**-17.4 ± 0.5^{c}**	-20.4 to -13.4
D-Ala10	16	**-15.5 ± 1.2^{d}**	-22.7 to -8.0

Despite the increased supercooling capacity of ND-Ala10 larvae (Table 7.2), the mean SCP remained approximately 5°C above that of control diapause larvae (D-Ala0) (Table 7.2).

7.4.1.4 Acute cold tolerance

Supplementing non-diapause larvae with alanine had a significant effect on their ability to recover from 2 h exposure to -10°C ($F_{3,240}$ = 5.76, P<0.005) (3)

Table 7.3 The effect of dietary alanine on mean) survival (±1 S. E. M.) of *C. vicina* non-diapause larvae following 2 h exposure to -10°C. Larvae were developed on 0 g (ND Ala0), 1 g (ND Ala1), 5 g (ND Ala5) or 10 g (ND Ala10) of alanine per 100 g mince. Diapause larvae are included for comparison. Means followed by different letters and in bold are significantly different from controls based on GLM *post-hoc* Bonferroni (P<0.05).

Treatment group	N	Survival ± 1 S. E. M. (%)	Range (%)
ND Ala0 (Control)	60	20.0 ± 0.5 [a]	10 – 40
ND Ala1	60	18.3 ± 0.6 [a]	0 – 40
ND Ala5	60	48.3 ± 0.5 [a,b]	30 - 70
ND Ala10	60	**51.7 ± 1.1[b]**	20 - 90
D Ala0 (for reference only)	60	36.7 ± 0	20 - 70

7.4.1.5 Chronic cold tolerance

The chronic cold tolerance of non-diapause larvae was determined as the time taken to induce 10% (LT10) and 50% (LT50) mortality at -4°C and -8°C (Figure 7.3 and Table 7.4). Dietary alanine significantly increased LT_{50} but not LT_{10} survival at -4°C for ND-Ala5 and ND-Ala10 larvae, as determined by non-overlapping Fiducial limits (4).

An increase in survival associated with increased dietary alanine was also observed for non-diapause larvae at -8°C. Survival was increased for all alanine-augmented larvae compared to ND-Ala0, with the exception of the LT_{10} for ND-Ala1.

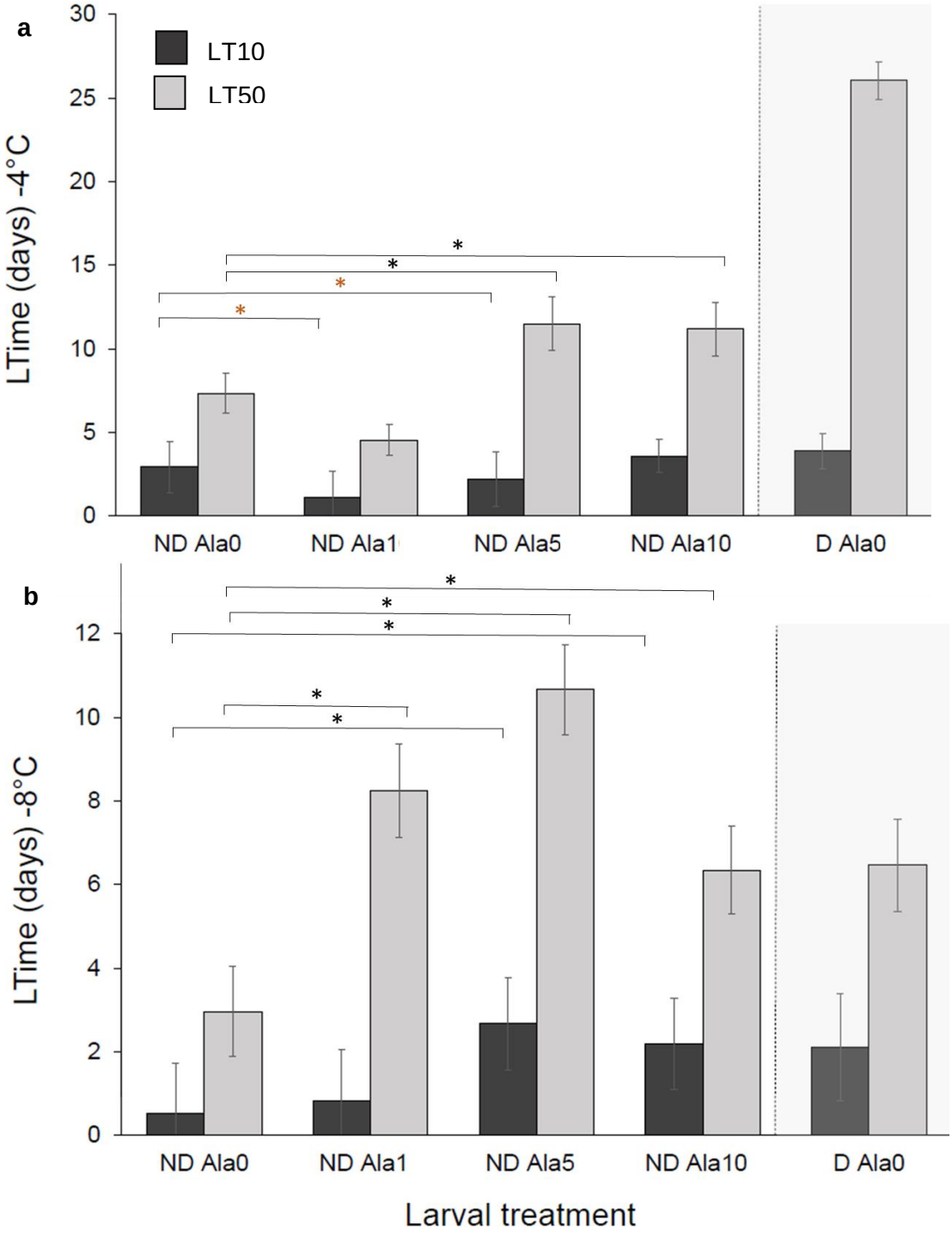


Figure 7.3 The effect of dietary alanine on the mean time (± 95% Fiducial limits) taken to induce 10% (LT10) and 50% (LT50) mortality for (a) non-diapause larvae at -4°C and (b) -8°C. Larvae were developed on 0 g (ND-Ala0), 1 g (ND-Ala10), 5 g (ND-Ala50) or 10 g (ND-Ala100) of alanine per 100 g mince. D Ala0 is included for comparison. *n* = minimum of 180 individuals per treatment. Means significantly greater than control are marked with * and significantly lower with *.

Table 7.4 The Fiducial limits for the time taken to induce 10% (LT10) and 50% (LT50) mortality for non-diapause larvae held at -4°C and -8°C. Larvae were developed on 0 g (ND-Ala0), 1 g (ND-Ala1), 5 g (ND-Ala5) or 10 g (ND-Ala10) of alanine per 100 g mince. Means marked with different letters and in bold are significantly different from controls (D-Ala0).

Temp	**LTime**	**Treatment**			
		ND-Ala0	ND-Ala1	ND-Ala5	ND-Ala10
-4°C	LT10	2.9 (2.3 to 3.5) [a]	1.1 (0.7 to 1.6) [b]	2.2 (1.4 to 3.0) [a,b]	2.6 (2.3 to 4.3) [a]
	LT50	7.3 (6.6 to 8.1) [c]	4.5 (3.8 to 5.3) [d]	11.5 (9.7 to 14.3) [e]	11.2 (10.0 to 12.1) [e]
-8°C	LT10	0.5 (0.3 to 0.7) [f]	0.8 (0.5 to 1.2) [f]	2.7 (2.1 to 3.2) [g]	2.2 (1.8 to 2.5) [g]
	LT50	2.9 (2.5 to 3.4) [h]	8.2 (4.1 to 6.8) [i]	10.7 (9.2 to 12.6) [j]	6.3 (5.7 to 7.0) [i]

Diapause survival at -8°C was enhanced in D-Ala1 compared to D-Ala0 larvae, as shown by differences in LT10 and LT50 survival. However, survival was not enhanced in D-Ala5 or D-Ala10 when compared to D-Ala0 larvae (determined by non-overlapping 95% fiducial limits) (Figure 7.4 and 7.5).

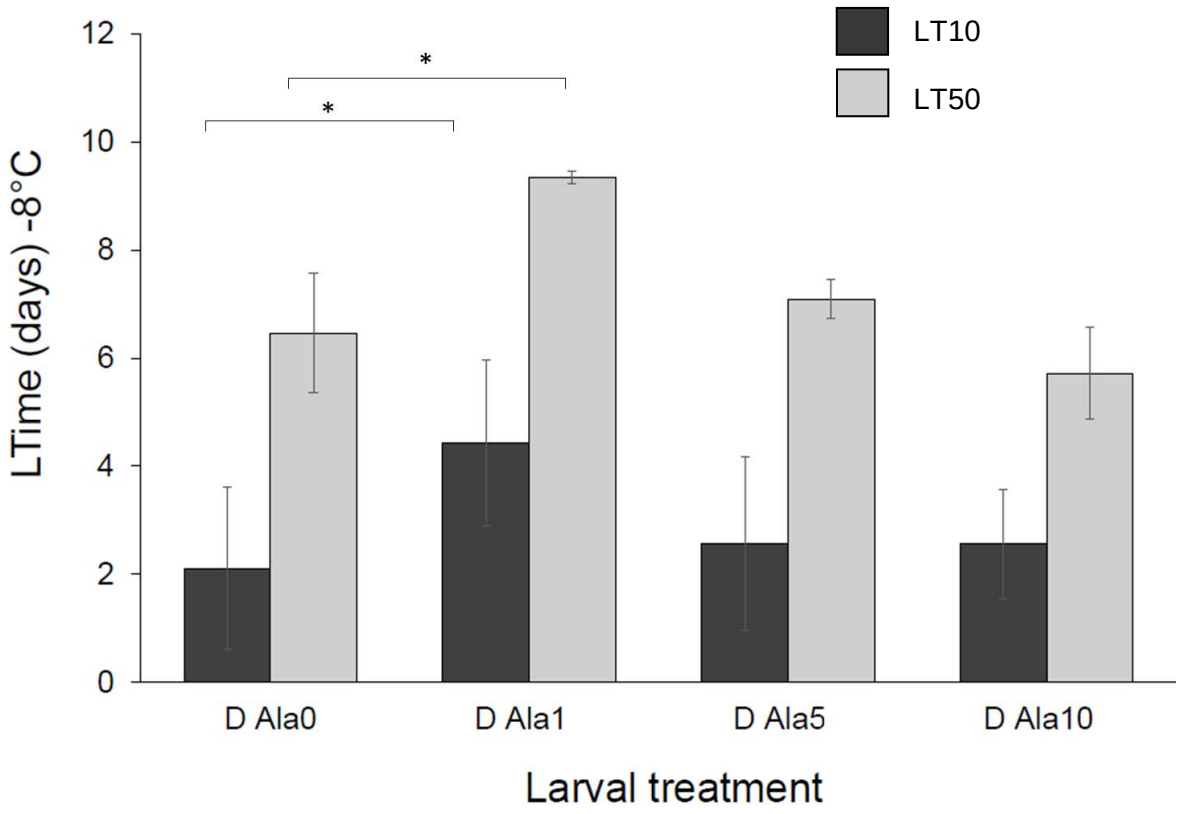


Figure 7.4 The effect of dietary alanine on the mean time (± 95% Fiducial limits) taken to induce 10% (LT10) and 50% (LT50) mortality for diapause larvae at -8°C. Larvae were developed on 0 g (D-Ala0), 1 g (D-Ala1), 5 g (D-Ala5) or 10 g (D-Ala10) of alanine per 100 g mince. *n* = minimum of 180 individuals per treatment. Significant differences from controls are marked *, these limits are presented in Table 7.5.

Table 7.5 The Fiducial limits for the time taken to induce 10% (LT10) and 50% (LT50) mortality for diapause larvae at held at -8°C. Larvae were developed on 0 g (D-Ala0), 1 g (D-Ala1), 5 g (D-Ala5) or 10 g (D-Ala10) of alanine per 100 g mince. Means marked with different letters and in bold are significantly different from controls (D-Ala0). To accompany Figure 7.4.

Temp	LTime	Treatment			
		D-Ala0 (Control)	**D-Ala1**	**D-Ala5**	**D-Ala10**
-8°C	LT10	2.1 (1.5 to 3.3) a	**4.4 (3.4 to 6.1) b**	2.6 (1.7 to 3.3) a	2.6 (1.6 to 3.3) a
	LT50	6.5 (5.1 to 7.5) c	**9.3 (7.9 to 11.3) d**	7.1 (6.7 to 7.3) c	5.7 (5.0 to 7.1) c

7.4.2 Adults

7.4.2.1 Adult survival

Alanine dissolved in water and provided directly to adults had a significant effect on survival ($F_{3,20}$ = 140.39, *P*<0.001), with 0% survival for adults provided 10 g alanine, 42.25 ± 1.9% survival for 5 g and 70.5 ± 0.7% survival for 1 g. Due to the high mortality experienced by adults provided with highest concentration of alanine, only those reared on 1 and 5 g of alanine were used for subsequent analysis.

There was no mortality in adults developed from ND-Ala10 larvae.

7.4.2.2 Supercooling capacity

Across all treatments, providing a dietary alanine supplement had a significant effect on adult supercooling capacity relative to untreated controls ($F_{3,38}$ = 6.34, *P*<0.001) (Table 7.6).

Table 7.6 The effect of dietary alanine on SCP (± 1 S.E.M) of adult *C. vicina*. Adults were either developed from larvae provided with alanine or provided with alanine during adult development. Means followed by a different letter and in bold are significantly different from each other based on GLM *post-hoc* Bonferroni (*P*<0.05).

Treatment	Life-stage treated	N	Mean ± SE (°C)	Range (°C)
None (control)	None	15	-8.8 ± 0.6 [a]	-14.0 to -5.6
1g alanine:100ml H_20	Adult	8	-10 ± 1.0 [a]	-13.8 to -6.4
5g alanine:100ml H_20	Adult	8	**-5.9 ± 0.5 [b]**	-9.3 to -5.0
10g alanine:100g mince	Larvae	11	-10 ± 0.6 [a]	-13.7 to -6.2

Post-hoc analysis determined this difference to be significant between adults provided directly with alanine and untreated control (*P*<0.05). No difference was detected in SCPs between all other treatments.

7.4.2.3 Chill coma and CT_{min}

Alanine supplementation had a significant effect on adult CT_{min} ($F_{3,38}$ = 16.89, *P*<0.001) and chill coma temperature ($F_{3,38}$ = 12.44, *P*<0.001) (Table 7.7).

Table 7.7 The effect of dietary alanine on mean (± 1 S.E.M.) CTmin and chill coma temperature of *C. vicina* adults with ranges (in brackets). Adults were either developed from larvae provided with alanine or provided with alanine post-eclosion. Means followed by different letters and in bold are significantly different from controls based on GLM *post-hoc* Bonferroni (*P*<0.05).

Treatment group	**Life stage provided with Alanine**	**N**	**CT_{min}**	**Chill coma**
None (control)	None	15	-2.5 ± 0.3 [a] (-4.5 to 1.1)	-6.5 ± 0.3 [b] (-9.7 to -5.1)
1 g alanine:100 ml H_20	Adult	8	-1.7 ± 0.3 [a] (-4.4 to 1.1)	**-8.6 ± 0.3 [c] (-11.3 to -6.2)**
5 g alanine:100 ml H_20	Adult	8	**4.8 ± 1.0 [b] (-1.3 to 10.7)**	-6.3 ± 0.2 [b] (-7.3 to -5.3)
10 g alanine:100 g mince	Larvae	11	-4.2 ± 0.8 [a] (-8.0 to 2.3)	**-8.9 ± 0.4 [c] (-10.9 to -6.5)**

Adults provided with alanine in their water had CT_{min} temperatures above the control group. *Post-hoc* analysis identified a significant difference between control adults and the 5 g:100 ml treatment group (*P*<0.001). There was no difference in CT_{min} between adults developed from larvae provided with dietary alanine and controls (*P*=1.00).

Chill coma temperatures were significantly lower than control adults for adults provided with an alanine:water content of 1 g:100 ml (*P*<0.001), and in adults developed from larvae provided with an alanine-augmented diet (*P* <0.001).

7.4.3 Metabolomic comparison

For the comparison between D, ND-Ala0 (the control) and ND-Ala5 (the supplemented larvae) four PC axes were generated, explaining 77.29% of the total variance (Figure 7.5). PC1 explained the majority of the variance (41.35%) (Figure 7.5a) and for this reason was the main focus of the data analysis. PC2 explained 16.64% of the variance (Figure 7.5b),

PC3 10.83% (Figure 7.5c) and PC4 8.47% (Figure 7.5d). For each loading plot D-Ala0 and ND-Ala0 samples are plotted in the positive region and ND-Ala5 in the negative region of Figure 7.5. The larger peaks indicate a greater contribution to the overall variance.

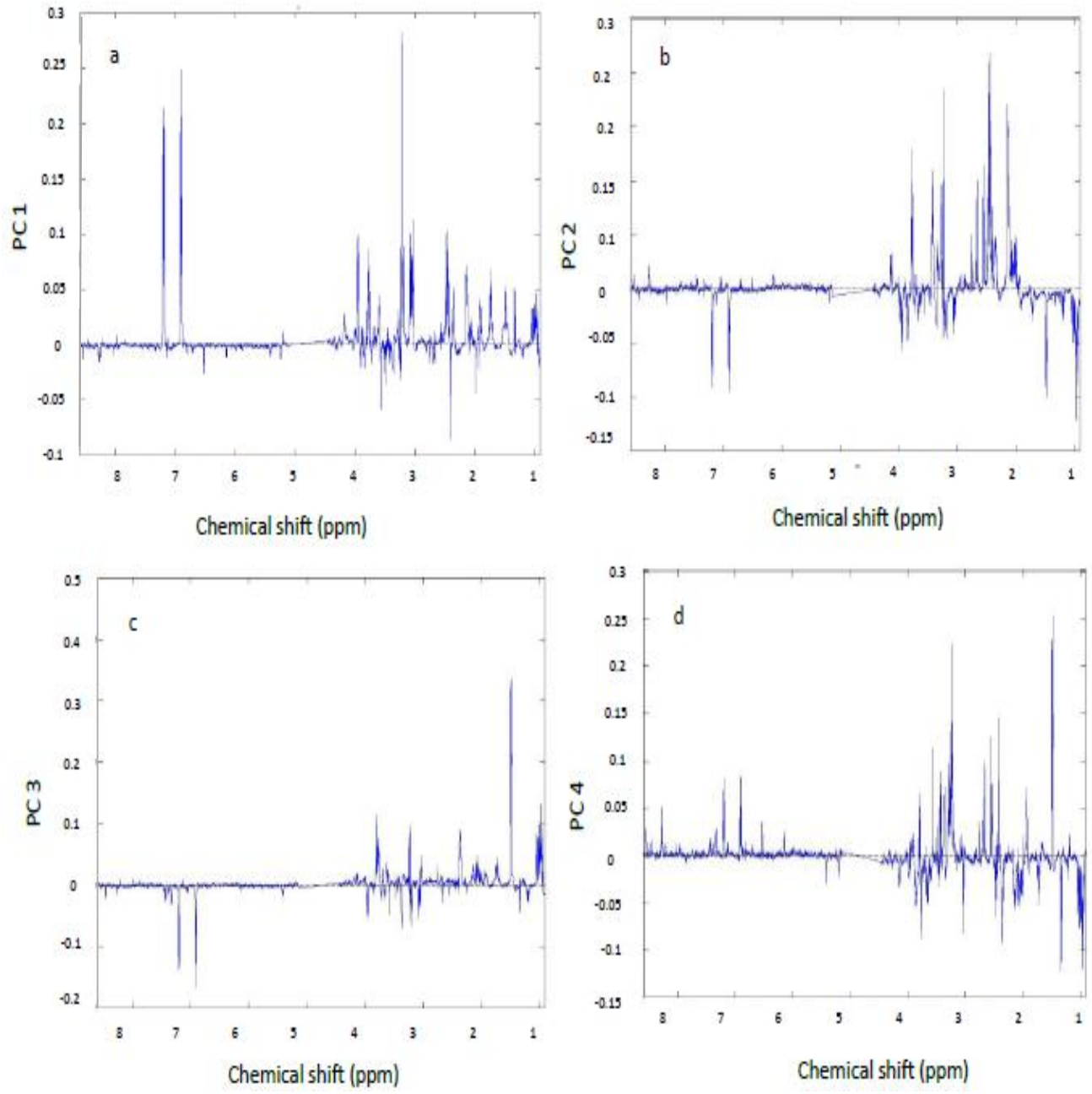


Figure 7.5 Loadings plot for (a) PC1 (b), PC2 (c), PC3 and (d) PC4 showing the key differences between the three sample groups. Metabolites more abundant in diapause and non-diapause controls are represented by positive peaks, while metabolites up-regulated in alanine-supplemented non-diapause larvae are represented by the negative peaks.

The PCA plot generated by the metabolomics analysis of all alanine supplemented larvae shows three distinct clusters for D-Ala0, ND-Ala0 and ND-Ala5 larvae (Figure 7.6). The clusters are clearly separated with the exception of one outlier for D-Ala0. PC 1 explains 41.4% of the variance in the dataset. As the main focus of this chapter is the effect of providing alanine to non-diapause larvae subsequent analysis was performed with D-Ala0 excluded.

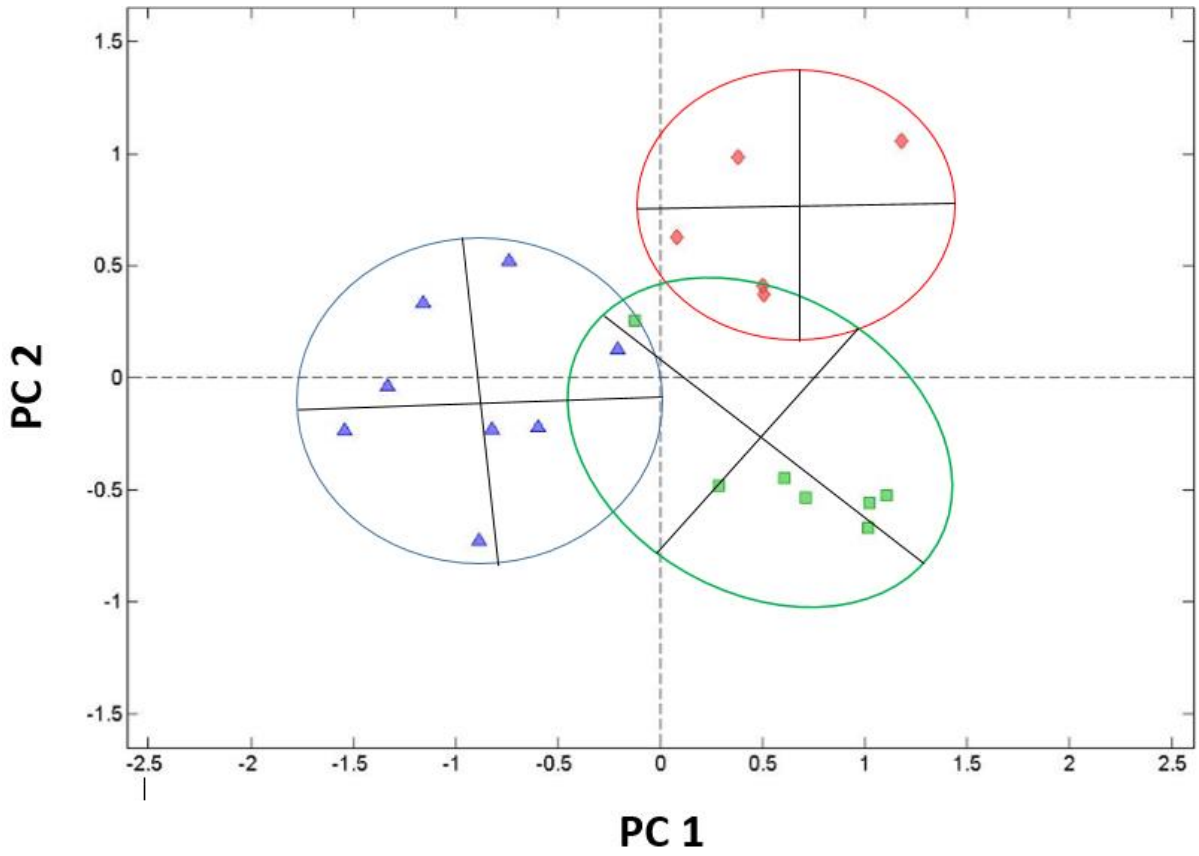


Figure 7.6 PC1 versus PC2 scores plot for the spectra of *C. vicina* diapause larvae (green, *N* =*n* = 7), non-diapause larvae (red, *n* = 5) and non-diapause larvae provided with a dietary source of an alanine specific dose (blue, *n* = 8)

For the comparison between control (ND-Ala0) and supplemented (ND-Ala5) larvae 3 PC axes were generated, explaining 76.12% of the total variance. Additional PCs were not generated as they would not change the values attributed to PC1 and PC2. PC1 explained the majority (53.05%) of the variance (Figure 7.7a). PC2 explained 11.97% of the variance (Figure 7.7b) and PC3 11.10% (Figure 7.7c). PC1 was the main focus of the analysis as it

explained over 50% of the total variance. For each loading ND-Ala controls are plotted in the positive region and ND-Ala5 are in the negative region of Figure 7.7. The larger peaks indicate a greater contribution to the variance. PC 1 explains the most variance so this was the main focus of the analysis.

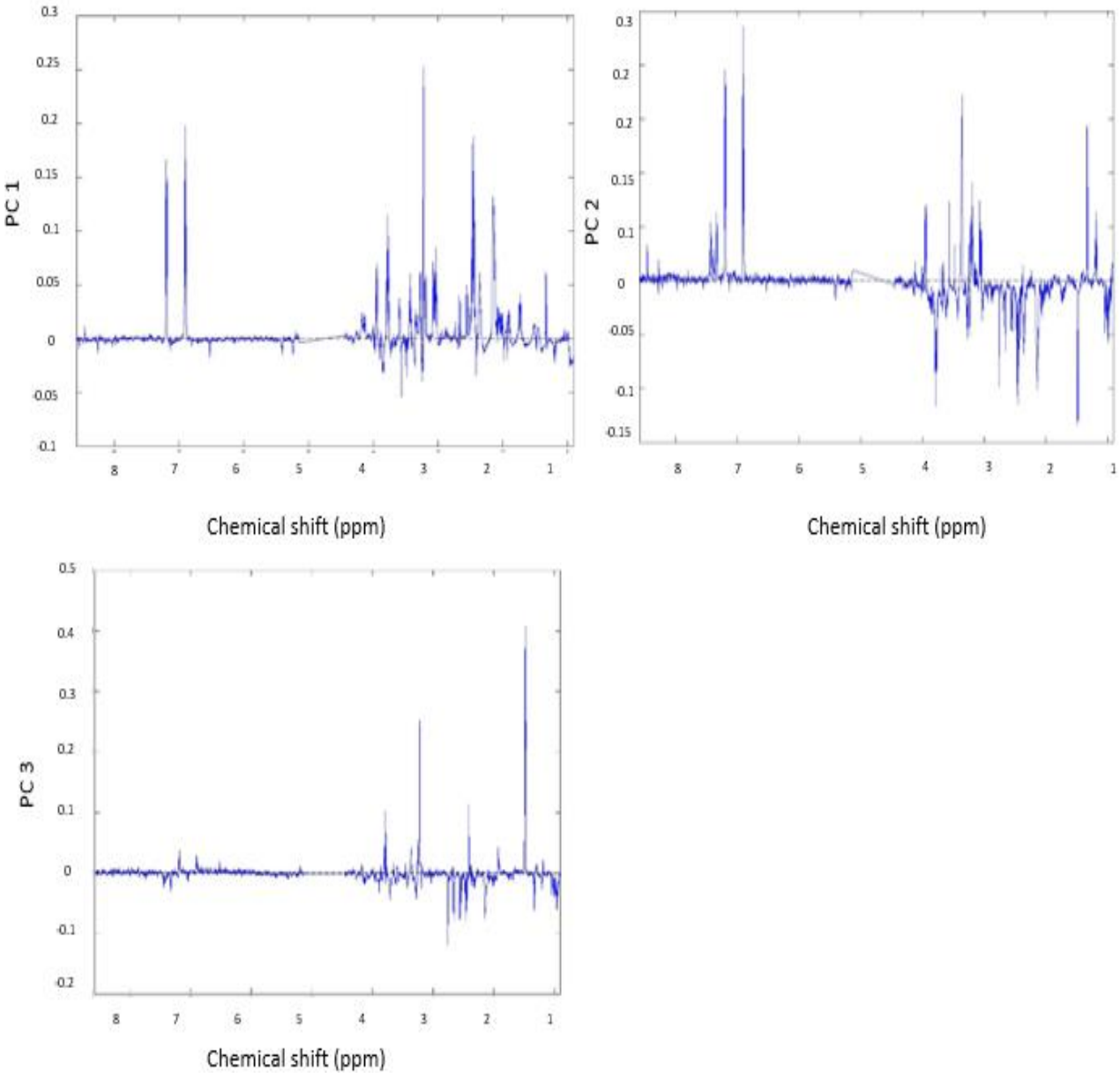


Figure 7.7 Loadings plots for (a) PC1 (b), PC2 and (c) PC3 showing the key differences between the non-diapause supplemented and non-diapause sample groups. Metabolites more abundant in diapause supplemented larvae are represented by positive peaks, while metabolites up-regulated in non-diapause larvae are represented by the negative peaks.

The broad-scale differences generated by the metabolomics analysis for ND-Ala0 and ND-Ala5 larvae are presented in Figure 7.8. The PCA clusters appear unambiguous with distinct groups for ND-Ala0 (control) and ND-Ala5 samples. These differences are represented more clearly as fold changes in Figure 7.9.

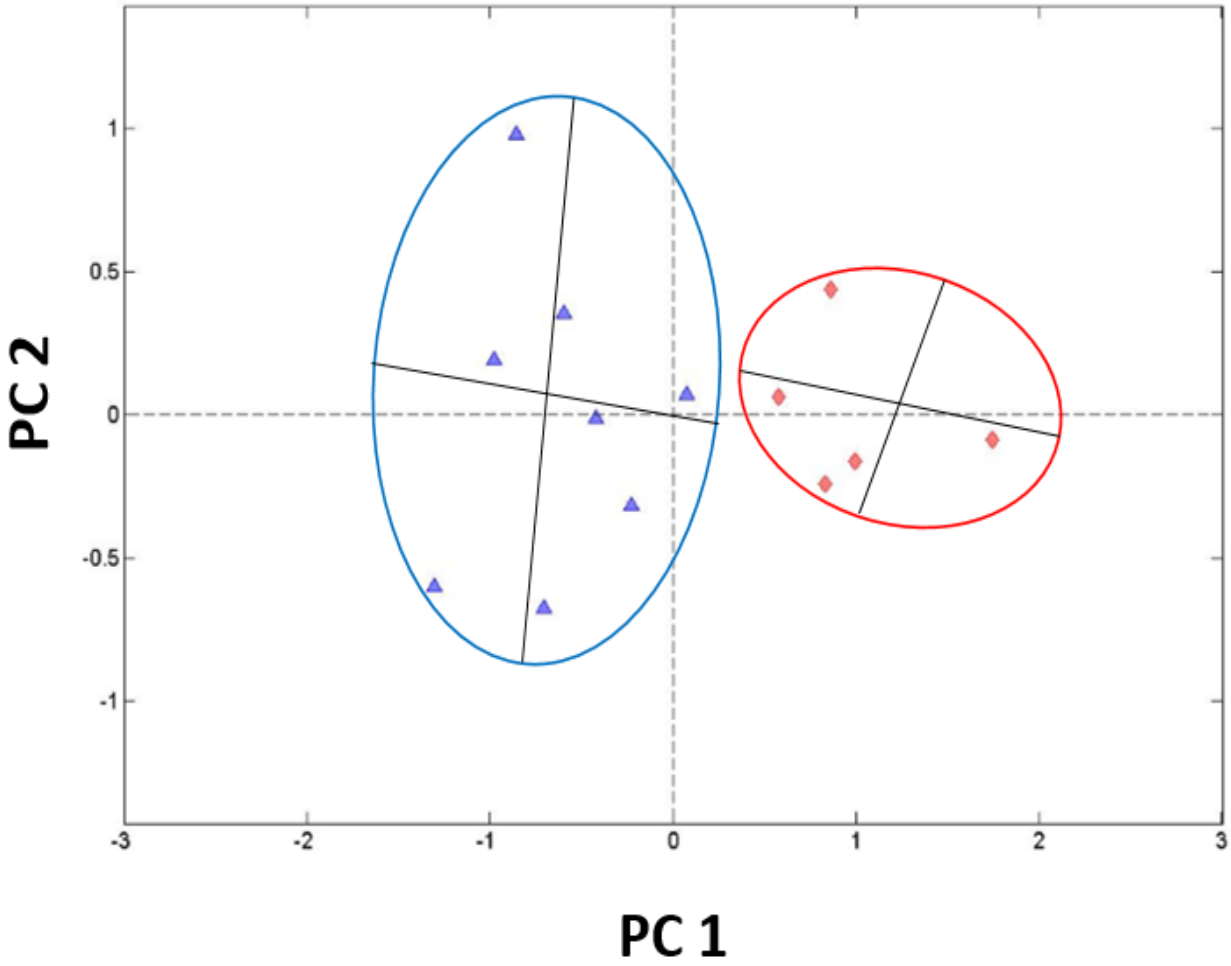


Figure 7.8 PC1 versus PC2 scores plot for the spectra for *C. vicina* non-diapause larvae (red, *n* = 5), non-diapause supplemented larvae (blue *n* = 8).

The three metabolites most strongly up-regulated in ND-Ala5 were succinate (0.95% fold change), Glycine (0.74% fold change) and Tyramide (0.67% fold change) (Figure 7.9).

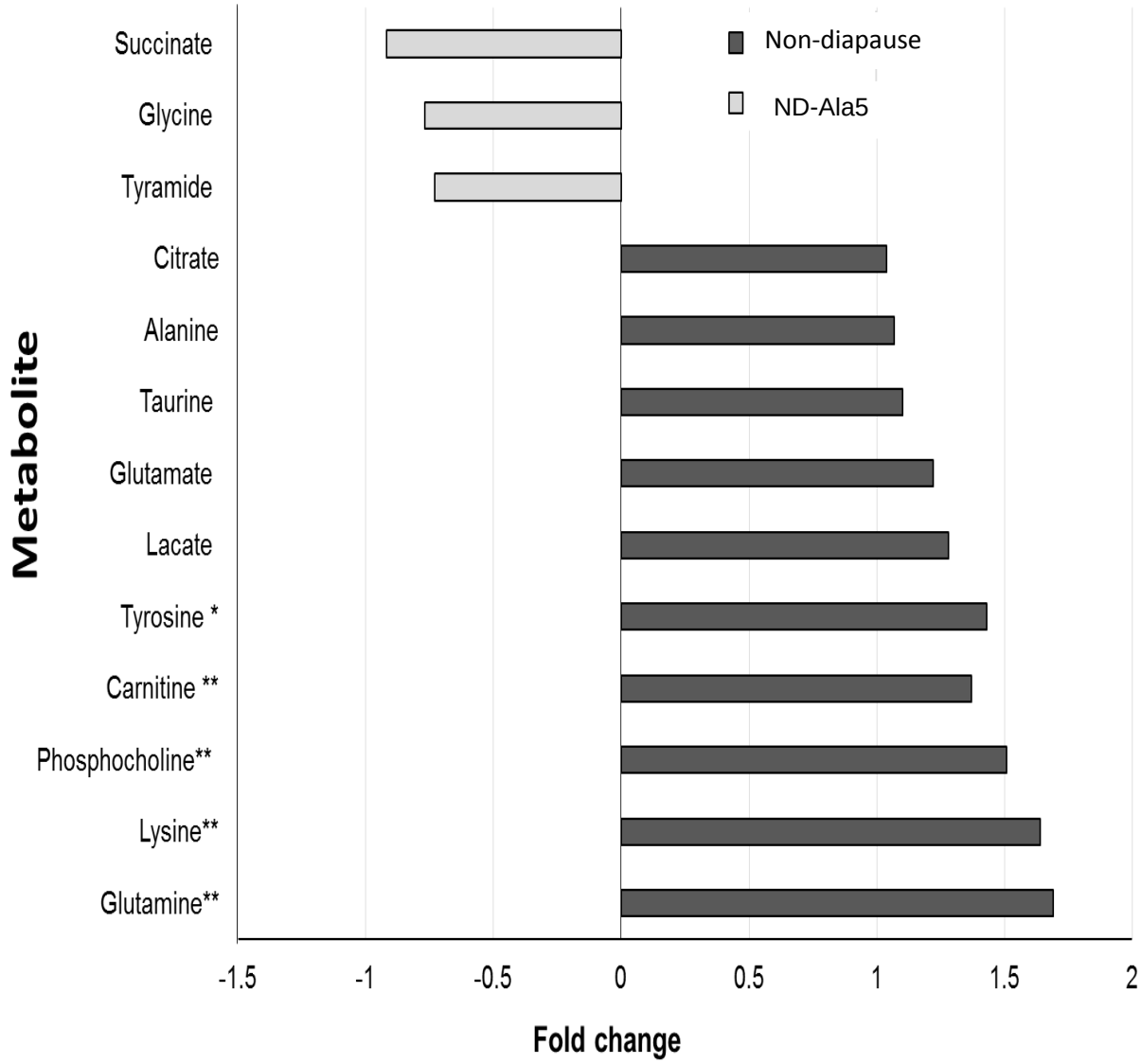


Figure 7.9 Comparison of 13 metabolites demonstrating the greatest differences between *C. vicina* non-diapause controls (dark grey) and non-diapause larvae provided with a dietary source of alanine (light grey) after 1H NMR analysis. Fold change is calculates as the mean content of the metabolite in diapause larvae divided by non-diapause larvae. * designates metabolite significant to $P<0.05$ and ** designates metabolite significant to $P<0.001$ between treatments

7.5 Discussion

Previous studies have identified changes in the abundance of amino acids, to be a key component of the diapause programme (Michaud and Denlinger, 2007; Colinet et al., 2012; Johnson, 2013), and in organismal responses to cold (Koštál et al., 2011a,b, 2012). In *C.*

vicina, alanine and proline were both identified as being up-regulated during diapause. However, it was unclear whether the accumulation of amino acids was part of a cold protection response, or if they accumulate as a by-product of other physiological adaptations, such as alanine synthesis in response to glycolysis (Johnson, 2013). To investigate the role of amino acids within the diapause programme, non-diapause larvae were provided with a dietary source of either proline or alanine, and the expression of diapause-associated phenotypes (slowed development, increased supercooling capacity and cold tolerance) was subsequently investigated. However, provision of a proline-augmented diet produced high levels of mortality in both diapause and non-diapause larvae (Table 7.1). The same dietary concentrations were provided to *C. costata* and *D. melanogaster*, with no increase in mortality (Kostál et al., 2011b; Koštál et al., 2012).

The incongruous effect of dietary proline may in part be explained by the metabolomic profiling of *C. costata* and *D. melanogaster*, which found proline to accumulate naturally to high levels in cold-hardy life stages (around a seven fold increase for both). A metabolomic profiling of *C. vicina* found only a moderate increase in proline during diapause (i.e. 1.12-fold increase) (Johnson, 2013). The role of proline as a cryoprotective compound appears to be species-specific, with increased cold tolerance in proline-enriched *C. costata* and *D. melanogaster* (Kostál et al., 2011b,c; , 2012) and reduced levels of proline in cold-hardy life stages of the mealworm, *Alphitobius diaperinus* (Lalouette et al., 2007). Given the high levels of mortality and its low level accumulation during diapause, it is unlikely that proline has a role in cryoprotection for *C. vicina*. Nevertheless, this does not suggest that proline does not play an important role in the cold protection of other species (Kostál et al., 2011a,c, 2012).

Diapause and non-diapause larvae developed on the alanine-enriched diet did not display adverse side-effects, with low levels of mortality across all treatment groups (Table 7.1). The high survival rate further substantiates the understanding of alanine as a non-toxic compound which often accumulates to high levels in overwintering life stages (Michaud and Denlinger, 2007). This does, however, contrast with the high levels of mortality experienced

by adults provided with dietary alanine. It is unclear why alanine enrichment would induce high levels of mortality in adults but not in larval life stages. One possible explanation is that alanine supplementation created an osmotic imbalance in adult *C. vicina*, which may not naturally produce high levels of alanine, resulting in cell dehydration and mortality. This is supported by Musselman et al. (2011), who found that larval *D. melanogaster* reared on high-sugar diets demonstrated a loss of metabolic homeostasis.

The first diapause-associated phenotype to be investigated in non-diapause supplemented larvae was development time (Figure 7.2). Diapause in *C. vicina* is associated with extended time as third instar-larvae and a delay in the timing of pupariation (Johnson, 2013). At 11°C, diapause larvae will remain as third-instar larvae for approximately 50 d, while for non-diapause larvae it is under 10 d. Dietary alanine had the effect of delaying development in non-diapause larvae (ND-Ala10, ND-Ala50 and ND-Ala100) when compared to larvae developed on the control diet (ND-Ala0) (Figure 7.2). This was a cumulative response, with an increase in the time from oviposition to mass wandering, mass wandering to pupariation and from pupariation to adult eclosion. However, the time spent as third-instar larvae was considerably shorter than that for diapause larvae, with a difference of approximately 42 d. Previous studies have associated sucrose, fructose, glucose and trehalose-enrichment with delayed development in *D. melanogaster* (Colinet et al., 2013), suggesting that delayed development could be a widespread response to dietary manipulation brought about by a nutritional imbalance (Musselman et al., 2011) and not necessarily indicative of expressing a diapause phenotype.

The second diapause-associated phenotype to be investigated was supercooling capacity, with lower SCPs associated with diapause for a number of species, including the codling moth, *Cydia pomonella* (Rozsypal et al., 2013), the leaf beetle, *Aulacophora nigripennis* (Watanabe and Tanaka, 1998) and *C. vicina* (Johnson, 2013). Previous chapters have identified *C. vicina* diapausing larvae to have a mean SCP of -18.9°C, compared to -11.4°C, for non-diapause larvae (Figure 2.2). The SCP of non-diapause larvae was lowered by 3.3°C

following development on an alanine-enriched diet (ND-Ala10). While this was 5°C above the mean SCP of diapause larvae, it provides the first direct evidence that development on an alanine diet has the capacity to increase supercooling capacity. It is important to recognise that SCP temperature represents the physiological limit of supercooling, and not the ecological limit of survival (Bale, 1996; Renault et al., 2002). For this reason, the effect of alanine augmentation on cold tolerance was also investigated. The cold tolerance of non-diapause supplemented larvae was investigated as their ability to recover from acute exposure (2 h exposure to -10°C) and chronic exposure to -4°C and -8°C. Chapters 2 and 3 found non-diapause larvae to be less tolerant to acute and chronic cold exposure than their diapause counterparts. Non-diapause larval development on an alanine diet increased survival following 2h exposure to -10°C by up to 31.7% (ND-Ala10) (Table 7.3), and the time taken to induce 50% mortality at -4°C was increased by 4.2 d (ND-Ala5) and 7.8 d at -8°C (ND-Ala5) (Figure 7.3 a and b). The acute cold tolerance of ND-Ala5 and ND-Ala10 larvae, and the chronic cold tolerance of ND-Ala1 and ND-Ala5 (at -8°C), actually exceeded that of diapause larvae (Table 7.3 and Figure 7.3a and b). An increase in cold hardiness is a widely reported response to entering diapause (Goto et al., 2001; Koštál, 2006; Hahn and Denlinger, 2011; Khodayari et al., 2013) associated with synthesis of blood sugars and polyols (Hahn and Denlinger, 2011), anti-freeze proteins, HSPs (Rinehart et al., 2007), and alterations to the lipid membrane bilayer (Michaud and Denlinger, 2006). However, the effect of dietary supplementation in enhancing the cold tolerance of non-diapause life stages supports previous studies which have confirmed that amino acids have a possible role in insect cryoprotection (Koštál et al., 2011c, 2012).

Alanine has been associated with increased cold tolerance in the Antarctic midge, *B. antarctica* (Michaud et al., 2008) and has been found to accumulate in cold-hardy life stages of the fall webworm, *H. cunea* (Li et al., 2001). Additionally, *O. furnacalis* (Goto et al., 2001) removed from the wild during periods of low temperature display increased levels of alanine, although a relationship between cold tolerance and alanine was not confirmed. It is believed

that alanine is able to help stabilise proteins at low temperatures (Carpenter and Crowe, 1988). Thereby, reducing the potential of low temperature mortality. However, relatively little is known about the direct role of alanine in enhancing low temperature tolerance.

Developing diapause larvae on an alanine-enriched diet also had an effect on the supercooling capacity and survival following chronic cold tolerance (Figure 7.4). However, this relationship followed that of a dose response curve. Diapause larvae provided with the lowest dose of dietary alanine (D-Ala1) displayed a moderate increase in chronic cold tolerance (Figure 7.4), while larvae developed on higher doses (D-Ala5 and D-Ala10), exhibited a weakened supercooling capacity (Table 7.2) and a decrease in chronic cold tolerance compared to D-Ala1 larvae (Figure 7.4). It is possible that diapause larvae are already approaching the physiological limits of cold tolerance, as was exhibited by a limited rapid cold hardening ability in Chapter 3, and thus there is limited additional physiological capacity to extend the limits of cold tolerance.

The effect of dietary manipulation in the larval life stage (adults developed from ND-Ala10 larvae) continued to be detected at the adult life stage, as demonstrated by a decrease in chill coma temperature of 2°C when compared to control adults (Table 7.7). This suggests that similar mechanisms may underpin cold tolerance across numerous life stages of *C. vicina*. Unlike adults developed from dietary enriched larvae, adults provided with a direct source of alanine experienced high levels of mortality (up to 100%). There was also a reduced supercooling capacity (up to 2.9°C) and they entered CT_{min} at higher temperatures (up to 7.3°C) than control adults (Table 7.6 and 7.7). A negative response between dietary manipulation and cold tolerance has previously been identified in *D. melanogaster* adults following larval enrichment with sucrose, fructose, glucose and trehalose (Colinet et al., 2013). It was suggested that dietary sugars produce a metabolic imbalance that can negatively affect cold tolerance in adult *D. melanogaster* (Colinet et al., 2013), a response that may have occurred in *C. vicina*.

The effect of dietary alanine on the adult life stages further substantiates the effect of protein feeding on reducing adult cold tolerance, as presented in Chapter 3. Adults accessing food lost the ability to express a RCH response and lost co-ordinated movement at higher temperatures. Feeding has also been found to impair chill coma recovery in the migratory locust, *Locusta migratoria* (Andersen et al., 2013). The findings presented here reinforce the suggestion in Chapter 3 that access to protein (of which amino acids are a predominant component), switches the development strategy to reproduction, with associated decreases in stress tolerance. Additionally, adults provided with a direct source of dietary alanine expressed a weakened supercooling capacity, which indicates that the alanine may be acting as a site of ice nucleation upon encountering low temperatures (Table 7.6).

The metabolomic comparison of ND-Ala0 (control) and ND-Ala5 larvae did not associate dietary manipulation with a significant increase in the content of free alanine. This result is not entirely surprising, as adult *D. melanogaster* developed on glucose and trehalose-enriched diets displayed only moderate increases in these compounds (Koštál et al., 2012; Colinet et al., 2013), yet, *D. melanogaster* displayed a 7.5-fold increase in proline content following development on a proline-enriched diet (Koštál et al., 2012).

ND-Ala5 larvae displayed increased levels of succinate, glycine and tyramide, while ND-Ala0 was mainly associated with increased glutamine, lysine, phosphocholine, canitine and tyrosine (Figure 7.9). The metabolites associated with ND-Ala5 are markedly different from those up-regulated during diapause (Figure 7.1) (Johnson, 2013) and it is unclear what role they could have in cold tolerance. Succinate is an intermediary of the Krebs cycle and has been detected in response to both cold and desiccation resistance in *B. antarctica* (Michaud et al., 2008). The accumulation in *B. antarctica* was taken to infer a decrease in Krebs cycle activity and increased glycolysis, a process which is believed to occur in diapausing *C. vicina* (Johnson, 2013). However, it is unlikely that dietary alanine has the capacity to instigate glycolysis and therefore increase the levels of succinate in non-diapause larvae. It is equally unlikely that glycine could have a role in cold hardiness as it was previously associated with

non-diapause larvae (Johnson, 2013) and there is no evidence from previous studies that tyramide has a role in cold tolerance.

Mine is the first study to conduct a metabolomic analysis following dietary supplementation of a potential cryoprotectant in an insect. These findings highlight that supplementation studies cannot automatically assume to increase the level of the metabolite concerned. Despite alanine not being significantly accumulated in treated larvae, it did have a clear effect on the phenotypes associated with diapause. The exact role of alanine in contributing towards the diapause phenotype appears to be complex and can only be surmised here. Alanine is known to combine with acetyl-CoA within the fat body for the synthesis of proline (Gäde and Auerswald, 2002; Arrese and Soulages, 2010), an amino acid which has been shown to increase cold tolerance in other insects (Kostál et al., 2011c). Alanine is also utilised in the production of glucose, which has also been identified as playing a role in cold protection (Michaud and Denlinger, 2007; Overgaard et al., 2007; Michaud et al., 2008; Hou et al., 2009). Neither proline nor glucose were up-regulated in the treated larvae. However, glucose is in turn utilised in the production of fatty acids, with modifications in the lipid membrane bilayer representing a key stress adaptation associated with diapause (Colinet et al., 2012). It is unclear whether dietary manipulation had an effect on the composition of the lipid membrane bilayer; however this could be an area of possible future research.

An important consideration for future metabolomics studies is that the information provided only delivers a snapshot of the metabolome. It is possible that earlier development stages would have displayed higher levels of alanine, following dietary manipulation, while the majority of alanine had been synthesised by the time of the metabolomic characterisation. Additionally, whole body extractions could miss tissue-specific responses. For example, a significant increase of alanine in the fat body (the site at which alanine is synthesised into proline and glucose) could be masked if alanine is not increased in other parts of the body.

7.6 Conclusions

In this chapter, I have shown that the provision of dietary alanine, during larval development, has the effect of delaying development. It also increased the supercooling capacity and enhanced the cold hardiness of non-diapause *C. vicina*. These are all phenotypes strongly associated with diapause, and suggest that alanine has an important role in the expression of cold hardiness mechanisms. Alanine has been associated with enhanced cold hardiness in previous studies, and is associated with an increased supercooling capacity in cold-tolerant life stages of *C. pomonella*. It is understood to stabilise proteins at low temperatures, and assist in the process of supercooling (Carpenter and Crowe, 1998; Michaud and Denlinger, 2007). However, the metabolomics comparison did not associate dietary manipulation with an accumulation of alanine, which makes its role in *C. vicina* unclear.

This chapter adds to a growing body of evidence, which suggests that amino acids play a role in insect cold tolerance. Previous studies have primarily focused on the role of proline (Kostál et al., 2011a,b,c, 2012). It is unlikely that proline contributes to increased cold tolerance during diapause for *C. vicina*. This suggests that amino acids may have a species-specific role in insect cold hardiness.

CHAPTER 8

GENERAL DISCUSSION

8.1 Overview

Climate change is currently the single greatest threat facing global biodiversity (Thomas et al., 2004). Understanding insect responses to climate change is of particular importance because of their fundamental role in providing ecosystem services, such as pollination and nutrient recycling (Losey and Vaughan, 2006), as well as representing key pests in agriculture (Zhang et al., 2007) and vectors of disease (Manachini et al., 2013; Oliveira et al., 2013; Shaw et al., 2013). A major limitation of current studies in predicting the impact of climate change on insect distributions and abundance is the tendency to focus on single life stage responses to higher temperatures (Nice and Fordyce, 2006; Mercader and Scriber, 2008; Zipkin et al., 2012). Cryptic life stages are often ignored, leaving the possibility that life stages vulnerable to the effects of environmental change, such as diapause, are going unnoticed, and responses to climate change unidentified. For example, the bog fritillary butterfly, *Boloria eunomia*, responds positively to warmer temperatures in most life stages (e.g. egg, pre-diapause larva and pupal survival). Higher temperatures are also associated with increased oviposition. Nevertheless, winter survival is significantly reduced under elevated temperatures (Radchuk et al., 2013). It is only through the careful consideration of both non-diapause and diapause developmental pathways that responses to climate change can be identified.

Many studies have furthered our understanding of insect responses to climate change (Crozier, 2003; Westgarthsmith et al., 2007; Terblanche et al., 2008; Jepsen et al., 2011; Musolin, 2012; Hoffmann et al., 2013). Yet, relatively little is known about how overwintering life stages are responding to environmental change (but see Bale and Hayward, 2010; Sgolastra et al., 2011; Radchuk et al., 2013). For temperate insects, the overwintering life stage must endure highly stressful environmental conditions. It is only through the implementation of specialised behavioural and physiological adaptations that winter survival is possible (Hahn and Denlinger, 2011). A core aim of this thesis was to employ *C. vicina* as

a ‘model’ system to further our understanding of the regulation of diapause, and the potential impact of future climate change.

8.2 Winter activity under continued climate change

Climate models predict that autumn temperatures will increase by up to 4°C over the course of this century, while winters will remain periods of prolonged cold (IPCC, 2013). In response to high Arctic warming, there is the potential of more frequent high and low temperature fluctuations over the winter months (Buckley and Kingsolver, 2012). For many temperate insects, this means that life stages active in the autumn, sensitive to diapause–induction cues, will be exposed to higher temperatures. While subsequent winter life stages will still encounter periods of chronic and acute cold.

Higher temperatures have been identified as the leading cause of species’ loss (Barnosky et al., 2011), range expansions (Musolin et al., 2010; Otaki et al., 2010; Walther, 2010) and alterations in the timing of yearly biological events (phenology) (Parmesan and Yohe, 2003; Visser and Both, 2005; Parmesan, 2006). This is not true just for insects, but a range of taxa with advancements in leaf emergence (Penuelas et al., 2002); egg laying onset in UK birds (Crick et al., 1997), and breeding in red squirrels, *Tamiasciurus hudsonicus* (Réale et al., 2003).

8.2.1 The adult life stage

The findings presented in this thesis have shown that for the blow fly, *C. vicina*, temperatures experienced by third-instar larvae are already causing the timing of diapause to be delayed beyond detection of the CDL. Higher temperatures are also associated with a reduced diapause incidence, and shortened diapause duration (Chapter 5). Under all scenarios, higher autumn and higher early-winter temperatures will disrupt the timing of diapause, leaving non-diapause life stages exposed to periods of winter cold. The findings presented in Chapter 4, indicate that adult life stages active over the winter months will reach CT_{min}, and

chill coma, at lower temperatures than summer-acclimated adults. Activity thresholds (CT_{min} and chill coma), were decreased following adult acclimation to lower temperatures, and culturing under a short-day photoperiod. Acclimation has been shown to have a similar effect on lowering activity thresholds for a number of species, such as *Syrphus ribesii* and *Myzus persicae* (Gibert et al., 2001; Hazell et al., 2010; Macmillan and Sinclair, 2011; Alford et al., 2012) and appears to be a well-conserved response. However, *C. vicina* is only the second species to show a lowering of activity thresholds (chill coma) in response to photoperiod (Vesala et al., 2012).

Unfortunately, it was not possible to determine whether the lowering of CT_{min} and chill coma was associated with the expression of an adult diapause. Further investigations could examine the effect of a short-day photoperiod on other cold tolerance mechanisms (LTime), and determine whether this is associated with delayed ovarian development. This is a known characteristic of adult diapause (Vinogradova, 1986).

8.2.2 Larval life stages and diapause

In addition to the direct effect of increased temperature acting on the adult life stage, the findings of this thesis have also identified cross generation responses, acting on larval progeny of the subsequent generation (Chapters 2, 5 and 6). Adults at higher temperatures produce fewer diapause progeny with a reduced diapause duration (Chapter 5). Warmer autumns are therefore likely to increase the ratio of non-diapause to diapause larvae. This response is believed to be widespread within the temperate zone (Mousseau and Dingle, 1991). Higher temperatures experienced by diapause programmed larvae can also cause diapause to be aborted (Chapter 5). Once diapause has been aborted, another life cycle must be completed to allow diapause to be initiated later in the year. Otherwise non-diapause life stages (larvae and adults) risk exposure to periods of sudden cold. It is therefore expected that the programming of diapause will be increasingly disrupted as climate change continues (Bale and Hayward, 2010). Indeed, field observations confirmed

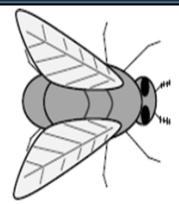
that diapause was delayed in years when average autumn temperatures were highest (Chapter 5). The delay in the programming of diapause was most closely associated with the frequency of soil temperatures above 15°C this being the threshold above which larvae are unable to initiate diapause. While there was no evidence to indicate that adults are currently experiencing diapause-aborting temperatures (25°C), this is increasingly likely as climate change continues.

One response to diapause disruption will be the exposure of non-diapause life stages to periods of chronic and acute winter cold. Based on the findings presented in Chapters 2, 4 and 6, it is known than non-diapause larvae are less cold-tolerant than their diapause counterparts, and therefore are more susceptible to cold-induced mortality (see Table 8.1 for a comparison).

While non-diapause larvae are less cold-tolerant than diapause larvae, they are still capable of tolerating temperatures typically experienced over the winter months. However, associated with the non-diapause pathway is a shortened development time. For example, at 5°C diapause larvae remain as L3 larvae for over 60 d (Chapter 5), while non-diapause larvae pupariate after approximately 12 d (Chapter 7). Non-diapause larvae are therefore more likely to resume development before favourable conditions have returned, a phenomenon that could be a widespread response to climate change (Bale and Hayward, 2010).

The shortened development time of non-diapause larvae increases the likelihood that development will be resumed before favourable conditions have returned. Temperature has a modifying effect on the programming of diapause for the majority of species (Mousseau and Dingle, 1991). Increased autumn temperatures pose the risk of disrupting the programming of diapause, disrupting synchrony with resource availability (food and water) and exposing non-diapause life stages to periods of sudden cold, all of which could result in heightened winter mortality. This response is unlikely to be limited to *C. vicina*.

Table 8.1 A comparison of the phenotypic variation in cold tolerance between diapause and non-diapause *C. vicina* larvae. Information is provided for diapause incidence, development time, supercooling points (SCPs), the lethal time 50% mortality (LT50), and temperature exposure inducing 90% mortality.

Diapause larvae	Phenotype	Non-diapause larvae
Adults at 15°C produce 81% diapause larvae, at 20°C they produce 65% diapause larvae		Adults at 15°C produce 19% non-diapause larvae, at 20°C they produce 35% non-diapause larvae
	If diapause larvae experience temperatures of above 15°C, diapause is aborted	
60 d	**Time at L3 stage**	12 d
-18.9°C	**Mean SCP**	-11.4°C
26.0 d	**LT50 at -4°C**	5.1 d
5.1 d	**LT50 at -8°C**	1.7 d
-11°C	**90% mortality (2h exposure)**	-10°C

8.2.3 Transgenerational cold tolerance

A previously unrecognised threat, presented in Chapters 2 and 6, is that while autumn temperatures may not always be sufficiently high to abort diapause, they may still influence the biological transmission of information from parents to progeny. This is summarised in Table 8.2

Table 8.2 The cross generation effect of parental acclimation temperature on the programming of diapause in L3 larval progeny.

Life stage	Phenotype	Average autumn temperature 15°C	Average autumn temperature 20°C	Conclusion
 Adult	CTmin	-1.8°C	0.9°C	Adults at the higher temperature reach CTmin and chill coma at higher temperatures. The presence of a RCH ability enhances the cold tolerance of all adults, although the RCH ability is lost upon feeding. Feeding also increases the temperature that adults enter chill coma.
	Chill coma	-5.7°C	-1.8°C	
	RCH (3h at 0°C pre-treatment)	66.7%	66.7%	
 Diapause larvae	SCP	-18.9°C	-15.3°C	Adults at the higher temperature produce fewer diapausing larvae and the duration of diapause is reduced. At larval temperatures above 15°C diapause is aborted completely, regardless of parental temperature. Adults at the higher temperature produce larvae with a lessened supercooling capacity and a weakened cold tolerance ability. This was evident in reduced mean SCPs, increased mortality following chronic cold exposure to temperatures experienced *in-situ* and under more extreme temperatures and also in the acute cold tolerance. The phenotypic adjustments observed were associated with reduced glucose content in the more cold hardy larvae
	LT50 at 0°C	33.5 d	23.0 d	
	LT50 at -4°C	26 d	11.4 d	
	LT50 at -8°C	5.1 d	3.0 d	
	2h exposure temp inducing 90% mortality	-11°C	-10°C	

This is the first evidence of a transgenerational effect of cold tolerance in an insect, although transgenerational effects of environmental variation have previously been documented (Marshall, 2008; Castro et al., 2013; Plautz et al., 2013; Suter and Widmer, 2013). The ecological relevance of this response is that adults under higher temperatures, as climate change continues, will give rise to diapausing larvae with a shortened diapause duration and reduced cold hardiness. Within the UK, it is likely that diapausing C. vicina larvae will still be sufficiently cold tolerant to survive the winter. However increased mortality could be observed at northern range boundaries.

It is unclear whether this cross generation response will be evident in other species. However, given the close association between this response and the programming of diapause, and the widespread reliance of temperate insects on a maternal-induced diapause, a similar phenomenon could certainly be present in other insects. For species

already encountering conditions approaching the lower thermal limits of survival, a cross generation reduction in cold tolerance could lead to high levels of winter mortality. This finding supports the theory of Chown et al. (2010), that phenotypic plasticity is only beneficial if an individual prepares for the direction of environmental change. In situations where higher autumn temperatures are followed by period of sudden cold, organisms with the greatest phenotypic plasticity may actually be most at risk of mortality and extinction. The beneficial acclimation hypothesis (Wilson and Franklin, 2002) suggests that the fitness of an individual is enhanced, if conditions are similar to those which they have previously experienced. Under this hypothesis, individuals may respond positively to climate change as they are progressively exposed to higher temperatures over multiple generations, with each generation gradually acclimating to the increasing temperature. However, Chown et al. (2010) suggest that phenotypic plasticity could also result in negative responses due to the unequal rate of warming across seasons. This could be of particular importance in the temperate zone, where late-autumn temperatures are predicted to increase at a greater rate than winter temperatures (IPCC, 2013). The effect of this will be that life stages active over the autumn will experience gradually higher temperatures, as climate change continues, while winter generations will still have to endure periods of acute and chronic cold (Chown et al., 2010). It is already known, for species such as *C. vicina*, that adults under higher temperatures will produce fewer diapausing offspring (Bale and Hayward, 2010); however, it is not known what other phenotypes could be influenced by higher autumn temperatures.

8.2.4 Rapid cold hardening

In chapter 3, it was shown that non-diapause life stages (larvae and un-fed adults) possess a strong RCH ability. This response could provide added protection during periods of acute cold exposure. This is not a substitute for the chronic cold tolerance mechanisms induced during diapause. Given the widespread occurrence of diapause, its sensitivity to increased temperature, and the presence of RCH in a substantial number of insect species investigated to date, it is likely that winter survival is already being enhanced through RCH within nature.

8.3 Relationship between supercooling and cold tolerance

This is often brought into question (Renault et al., 2002; Terblanche et al., 2011). The SCP represents the point of intracellular ice formation, the absolute lower limit of survival for truly freeze avoiding insects (Bale, 1996, 2002). At temperatures above the SCP an insect will often encounter direct and indirect chilling injury. Eventually, if temperatures do not increase they will encounter cold-induced mortality (Denlinger and Lee, 2010). There is variability in the capacity of insects to survive at temperatures above the SCP. Species such as the Autumnal moth, *Epirrita autumna*, encounter mortality at temperatures approaching the SCP. The house fly, *Musca domestica*, experiences complete mortality at temperatures close to 0°C (Bale, 1996, 2002).

Despite its recognition as a poor indicator of cold tolerance, SCPs continue to be measured in cold tolerance studies. Its popularity is partly due to it providing a useful comparative index between studies. The relationship between the SCP and LT_{50} (at -8°C), using data from different treatments of diapause and non-diapause larvae, is presented for *C. vicina* in Figure 8.1.

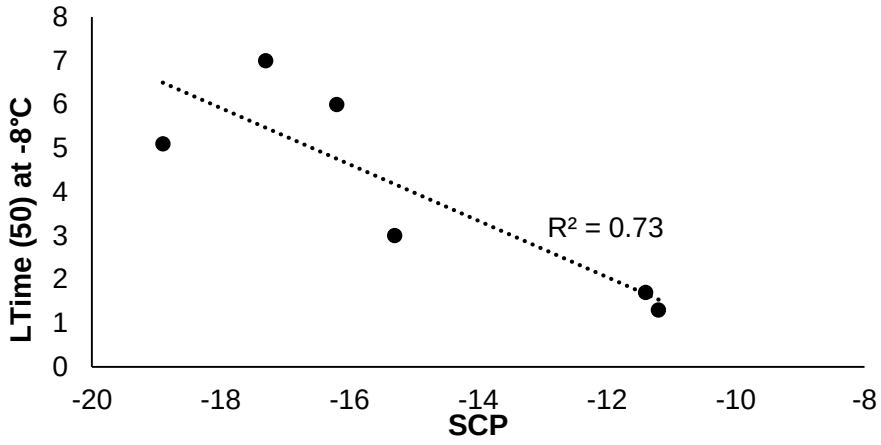


Figure 8.1 Model 2 regression and R^2 value for the relationship between the SCP and cold tolerance (LT_{50} at *-8°C).* The regression uses data collected for diapause and non-diapause life stages presented in Chapters 2 and 6.

For *C. vicina* larvae, therefore, there appears to be a strong correlation between SCPs and cold hardiness (R^2 = 0.73) across both diapause and non-diapause phenotypes. A relationship between SCPs and cold tolerance appears to be widespread, with a meta-analysis of over 350 freeze avoiding insects identifying a relationship between these two phenotypes (Turnock and Fields, 2005). A likely explanation for this, is that cryoprotectant compounds involved in cold protection, such as sugars and polyols, are also responsible for the inhibition of freezing at low temperatures. This is achieved through competing with water molecules to form hydrogen bonds at sites of ice nucleation (Zachariassen et al., 2004).

8.4 The link between cold tolerance and diapause

Previous work on *C. vicina* identified alanine as one of the dominant metabolites up-regulated during diapause (Johnson, 2013). Alanine is also associated with increased cold tolerance in a number of other species (Li et al., 2001; Michaud and Denlinger, 2007; Overgaard et al., 2007; Rivers et al., 2000). Evidence of a possible role in cold protection has been absent until now (Chapter 7). Non-diapause larvae, provided with an alanine-augmented diet, exhibited an increased supercooling capacity and heightened cold tolerance (Chapter 7). Subsequent metabolomic analysis did not associate alanine augmentation with increased levels of alanine. It therefore remains unclear what role alanine plays in the regulation of diapause and the associated increase in cold tolerance. Further investigation would be required to determine whether earlier life stages are accumulating alanine, or if alanine is being converted into other metabolites, with a role in cryoprotection. Interestingly, proline, which has been shown to heighten cold tolerance following dietary manipulation in other species, was not associated with increased cold tolerance (Koštál et al., 2011c, 2012). This suggests that the amino acids underpinning cold tolerance and/or diapause are species-specific.

The ability to heighten cold tolerance of non-diapause life stages, through alanine augmentation, confirms that metabolomic changes expressed during diapause, are involved

in heightening cold tolerance. Chapter 6 also confirmed a close association between diapause incidence, supercooling capacity and cold hardiness. This suggests that diapause is a pre-requisite for implementing seasonal increases in cold hardiness. This adresses the second aim of the thesis to investigate the relationship between diapause, and increased cold tolerance, and the role of amino acids in the expression of the cold hardiness phenotype.

8.5 Limitations and future direction

While this thesis has identified a transgenerational effect of parental culturing temperature on the cold tolerance phenotype, it has not been possible to ascertain what mechanisms are underpinning this response. There is good reason to assume that the transgenerational response, identified here, could be an epigenetic response. Epigenetic responses are physiological and behavioural changes occurring across generations, arising when environmental conditions, experienced by the adult generation, being about modifications to the phenology of their offspring (Ho and Burggren, 2010). Epigenetic responses are most notability induced by conditions experienced by the maternal generation (Burgess and Marshall, 2011). The response identified in Chapter 2 strongly correlated with maternal conditions.

The principal mechanisms involved in epigenetic processes are DNA methylation, chromatin induced gene silencing (Reinders et al., 2009) and altered RNA activity, modifying the function of specific proteins (Costa, 2008). This is an emerging area that I was unable to pursue during my PhD. To date, few studies have attempted to address the role of epigenetics in climate change responses (Burgess and Marshall, 2011; Salinas and Munch, 2012). There are an insufficient number of studies addressing epigenetics in a real-world context (Bossdorf et al., 2008). The response identified in this thesis provides the possibility of identifying an ecologically relevant epigenetic response.

Also identified in this thesis was the role of alanine supplementation in the expression of cold hardiness phenotypes within non-diapause larvae. Unfortunately, the response was not simply due to increased levels of alanine within non-diapause larvae. This leaves the role of alanine difficult to determine. Future studies could use RNA interference techniques to block the synthesis of alanine within diapause larvae, and identify whether this is associated with reduced cold hardiness. This could further substantiate its role as a cold protective compound. However, given the potential role of alanine in glycolysis, it is possible that the blocking of alanine synthesis, could also lead to mortality within diapause larvae which rely on glycolysis as the primary source of energy production (Johnson, 2013).

Furthermore, there is a general need for future research to establish the role of amino acids in insect diapause. To date, research has primarily focused on alanine (Chapter 7) and proline (Kostál et al., 2011a,b,c, 2012). However if amino acids play a ubiqituous role in cold tolerance, it is likely that additional amino acids contribute towards low temperature survival in other insects.

An additional area of insect diapause that requires further investigation is the role of the fat body in the regulation of diapause. Recent studies suggest, that gene expression within the fat body is responsible for inducing and terminating diapause. This is only just starting to emerge as a novel area of insect biology (Xu et al., 2012). The initiation of diapause is also associated with changes in the composition of the fatty acid bilayer. It is not yet known whether this is an integral part of diapause for *C. vicina*. It had been my intention to compare the lipid profile between diapause and non-diapause life stages, to determine the role of lipid metabolism during the regulation of diapause. A lipidomic analysis has been performed on diapause and non-diapause larvae but there was not the opportunity to analyse these data during my PhD.

The usefulness of *C. vicina* as a model species in investigating insect diapause, would benefit greatly from whole genome sequencing. As genome sequencing becomes increasingly cost-effective, it is likely that this will be done in the near future. Genome

sequencing would help to identify the processes involved in the programming of diapause, the expression of cold tolerance phenotypes and the transgenerational effect of parental temperature.

Furthermore, there is detailed understanding of the day degree development requirements for *C. vicina*, from forensic studies. This wealth of literature is seldom utilised within the field of insect cold tolerance. This information could be used to explain changes in seasonal phenology, development under different conditions and the environmental factors involved in inducing and terminating diapause.

Finally, while the majority of species enter a winter dormancy, virtually nothing is known about how most species will respond to winter climate change. An urgent area of future research is to identify how important pollinator species, such as bees, wasps, hoverflies, damselflies and dragonflies will respond to climate change. Increased winter mortality could have untold impacts on food production and ecosystem integrity. Future research must concentrate on identifying the possible loss of synchrony between environmental cues used to programme diapause (i.e. temperature and photoperiod), the effect of more variable winter temperatures on winter mortality, the widespread occurrence of a cross generation reduction in cold tolerance, and the loss of synchrony between spring emergence and resource availability.

In conclusion, *C. vicina* has proved to be an ideal model insect for investigations of insect diapause. Within this thesis, *C. vicina* has been used to identify a novel transgenerational response to temperature, previously unrecognised in any insect species. A combination of laboratory and field-based studies has identified the effect of elevated temperatures in disrupting the induction of diapause. Indeed, field-based evidence indicates that soil temperatures are already causing diapause to be delayed beyond its photoperiodic induction. Given that similar mechanisms (temperature and photoperiod) are involved in regulating diapause for the majority of temperate insects, the responses to climate change identified here may be applicable to temperate insects in general. Finally, *C. vicina* has proved to be a

useful species in further substantiating the link between environmental conditions, diapause and the implementation of cold tolerance mechanisms. It has also contributed towards recent studies which suggest that amino acids play an important role in insect cold tolerance.

REFERENCES

Adedokun, T. A. and Denlinger, D. L. (1985). Metabolic reserves associated with pupal diapause in the flesh fly, *Sarcophaga crassipalpis*. *Journal of Insect Physiology* **31**, 229–233.

Alford, L., Blackburn, T. M. and Bale, J. S. (2012). Effects of acclimation and latitude on the activity thresholds of the aphid *Myzus persicae* in Europe. *Journal of Applied Entomology* **136**, 332–346.

Alley, R. B., Berntsen, T., Bindoff, N. L., Chen, Z., Chidthaisong, A., Friedlingstein, P., Gregory, J. M., Hegerl, G. C., Heimann, M., Hewitson, B., et al. (2007). *A report for Working Group 1 of the Intergovernmental Panel on Climate Change. Summary for Policymakers*. Cambridge University Press, Cambridge UK.

Altermatt, F. (2012). Temperature-related shifts in butterfly phenology depend on the habitat. *Global Change Biology* **18**, 2429–2438.

Andersen, J. L., Findsen, A. and Overgaard, J. (2013). Feeding impairs chill coma recovery in the migratory locust (*Locusta migratoria*). *Journal of Insect Physiology* **59**, 1041–1048.

Anderson, J. F. (1968). Influence of photoperiod and temperature on the induction of diapause in *Aedes atropalpus*. *Entomologia Experimentalis et Applicata* **11**, 321–330.

Angilletta, M. J. (2009). *Thermal adaptation: a theoretical and empirical synthesis*. Cambridge University Press, Cambridge, UK.

Ansart, A. and Vernon, P. (2003). Cold hardiness in molluscs. *Acta Oecologica* **24**, 95–102.

Arrese, E. L. and Soulages, J. L. (2010). Insect fat body: energy, metabolism, and regulation. *Annual Review of Entomology* **55**, 207–225.

Bacastow, R. (1976). Modulation of atmospheric carbon dioxide by the Southern Oscillation. *Nature* **261**, 116–118.

Bale, J. S. (1987). Insect cold hardiness: Freezing and supercooling - an ecophysiological perspective. *Journal of Insect Physiology* **33**, 899–908.

Bale, J. S. (1993). Classes of insect cold hardiness. *Functional Ecology* **7**, 751–753.

Bale, J. S. (1996). Insect cold hardiness: A matter of life and death. *European Journal of Entomology* **93**, 369–382.

Bale, J. S. (2002). Insects and low temperatures: from molecular biology to distributions and abundance. *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences* **357**, 849–862.

Bale, J. S. and Hayward, S. A. L. (2010). Insect overwintering in a changing climate. *The Journal of Experimental Biology* **213**, 980–994.

Bale, J. S., O'Doherty, R., Atkinson, H. J. and Stevenson, R. A. (1984). An automatic thermoelectric cooling method and computer-based recording system for supercooling point studies on small invertebrates. *Cryobiology* **21**, 340–347.

Bale, J. S., Masters, G. J., Hodkinson, I. D., Awmack, C., Bezemer, T. M., Brown, V. K., Butterfield, J., Buse, A., Coulson, J. C., Farrar, J. (2002). Herbivory in global climate change research: direct effects of rising temperature on insect herbivores. *Global Change Biology* **8**, 1–16.

Barnosky, A. D., Matzke, N., Tomiya, S., Wogan, G. O. U., Swartz, B., Quental, T. B., Marshall, C., McGuire, J. L., Lindsey, E. L., Maguire, K. C. (2011). Has the Earth's sixth mass extinction already arrived? *Nature* **471**, 51–57.

Bartomeus, I., Ascher, J. S., Wagner, D., Danforth, B. N., Colla, S., Kornbluth, S. and Winfree, R. (2011). Climate-associated phenological advances in bee pollinators and bee-pollinated plants. *Proceedings of the National Academy of Sciences of the United States of America* **108**, 20645–20649.

Beach, R. (1978). The required day number and timely induction of diapause in geographic strains of the mosquito *Aedes atropalpus*. *Journal of Insect Physiology* **24**, 449–455.

Bellard, C., Bertelsmeier, C., Leadley, P., Thuiller, W. and Courchamp, F. (2012). Impacts of climate change on the future of biodiversity. *Ecology Letters* **15**, 365–377.

Block, W. (1982). Supercooling points of insects and mites on the Antarctic Peninsula. *Ecological Entomology* **7**, 1–8.

Block, W., Baust, J. G., Franks, F., Johnston, I. A. and Bale, J. (1990). Cold tolerance of insects and other arthropods. *Philosophical Transactions of the Royal Society B: Biological Sciences* **326**, 613–633.

Bosch, J., Sgolastra, F. and Kemp, W. P. (2010). Timing of eclosion affects diapause development, fat body consumption and longevity in *Osmia lignaria*, a univoltine, adult-wintering solitary bee. *Journal of Insect Physiology* **56**, 1949–1957.

Bossdorf, O., Richards, C. L. and Pigliucci, M. (2008). Epigenetics for ecologists. *Ecology letters* **11**, 106–115.

Bradshaw, W. E. and Holzapfel, C. M. (2001). Genetic shift in photoperiodic response correlated with global warming. *Proceedings of the National Academy of Sciences of the United States of America* **98**, 14509–14511.

British Dragongly Soceity (2013). *Dragonfly recording network*. http://www.anisoptera.org

Brook, E. J. (2009). Atmospheric carbon footprints? *Nature Geoscience* **2**, 170–172.

Broufas, G. D. and Koveos, D. S. (2001). Rapid cold hardening in the predatory mite *Euseius (Amblyseius) finlandicus* (Acari: Phytoseiidae). *Journal of Insect Physiology* **47**, 699–708.

Buckley, L. and Kingsolver, J. (2012). Functional and phylogenetic approaches to forecasting species' responses to climate change. *Annual Review of Ecology, Evolution, and Systematics* **43**, 205–226.

Burgess, S. C. and Marshall, D. J. (2011). Temperature-induced maternal effects and environmental predictability. *The Journal of Experimental Biology* **214**, 2329–3236.

Bürgi, L. P. and Mills, N. J. (2010). Cold tolerance of the overwintering larval instars of light brown apple moth *Epiphyas postvittana*. *Journal of Insect Physiology* **56**, 1645–1650.

Butterfly Conservation (2013). *Recording and Monitoring*. http://www.bfly.org

BWARS (2013). *Bees, Wasps & Ants Recording Society*. http://www.bwars.com

Carpenter, J. F. and Crowe, J. H. (1988). The mechanism of cryoprotection of proteins by solutes. *Cryobiology* **25**, 244–255.

Carrasco, M. A, Buechler, S. A, Arnold, R. J., Sformo, T., Barnes, B. M. and Duman, J. G. (2012). Investigating the deep supercooling ability of an Alaskan beetle, *Cucujus clavipes puniceus*, via high throughput proteomics. *Journal of proteomics* **75**, 1220–34.

Castro, B. M., Moriuchi, K. S., Friesen, M. L., Badri, M., Nuzhdin, V., Strauss, S. Y., Cook, D. R. and Wettberg, E. Von (2013). Parental environments and interactions with conspecifics alter salinity tolerance of offspring in the annual *Medicago truncatula*. *Journal of Ecology* **101**, 1281–1287.

Cheng, L. (1985). Biology of Halobates (Heteroptera: *Gerridae*). *Annual Review of Entomology* **30**, 111–135.

Chernysh, S. I. and Gordja, N. A. (2012). The immune system of maggots of the blow fly (*Calliphora vicina*) as a source of medicinal drugs. *Journal of Evolutionary Biochemistry and Physiology* **47**, 524–533.

Chernysh, S. I., Simonenko, N. P. and Numata, H. (1995). Sensitive stage for the diapause-averting effect of high temperature in the blow fly, *Calliphora vicina* Robineau-Desvoidy (Diptera: Calliphoridae). *Applied Entomology and Zoology* **30**, 498–499.

Chown, S. L. (2001). Physiological variation in insects : hierarchical levels and implications hardiness. *Journal of Insect Physiology,* **47**, 649–660.

Chown, S. L. and Gaston, K. J. (1999). Exploring links between physiology and ecology at macro-scales: the role of respiratory metabolism in insects. *Biological Reviews of the Cambridge Philosophical Society* **74**, 87–120.

Chown, S. and Nicolson, S. W. (2004). *Insect Physiological Ecology: Mechanisms and Patterns*. Oxford University Press, Oxford, UK.

Chown, S., Hoffmann, A., Kristensen, T., Angilletta, M., Stenseth, N. and Pertoldi, C. (2010). Adapting to climate change: a perspective from evolutionary physiology. *Climate Research* **43**, 3–15.

Christian, K. and Morton, S. (1992). Extreme thermophilia in a Central Australian Ant, *Melophorus bagoti*. *Physiological Zoology* **65**, 885–905.

Churchill, T. A. and Storey, K. B. (1989). Metabolic consequences of rapid cycles of temperature change for freeze-avoiding vs freeze-tolerant insects. *Journal of Insect Physiology* **35**, 579–585.

Colinet, H. and Hoffmann, A. A. (2012). Comparing phenotypic effects and molecular correlates of developmental, gradual and rapid cold acclimation responses in *Drosophila melanogaster*. *Functional Ecology* **26**, 84–93.

Colinet, H., Renault, D., Charoy-Guével, B. and Com, E. (2012). Metabolic and proteomic profiling of diapause in the aphid parasitoid *Praon volucre*. *PLoS ONE* **7**, e32606.

Colinet, H., Larvor, V., Bical, R. and Renault, D. (2013). Dietary sugars affect cold tolerance of *Drosophila melanogaster*. *Metabolomics* **9**, 608–622.

Convey, P. (1996). Overwintering strategies of terrestrial invertebrate in Antarctica - the significance of flexibility in extremely seasonal environments. *European Journal of Entomology* **93**, 489–505.

Coombs, M. R. and Bale, J. S. (2013). Comparison of thermal activity thresholds of the spider mite predators *Phytoseiulus macropilis* and *Phytoseiulus persimilis* (Acari: Phytoseiidae). *Experimental & Applied Acarology* **59**, 435–445.

Costa, F. F. (2008). Non-coding RNAs, epigenetics and complexity. *Gene* **410**, 9–17.

Coulson, S. J. and Bale, J. S. (1990). Characterisation and limitations of the rapid cold-hardening response in the housefly *Musca domestica* (Diptera: Muscidae). *Journal of Insect Physiology* **36**, 207–211.

Cowles, R. and Bogert, C. M. (1944). A preliminary study of the thermal requirements of desert reptiles. *Bulletin of the American Museum of Natural History* **83**, 263–296.

Crick, H. Q. P., Dudley, C., Glue, D. E. and Thomson, D. L. (1997). UK birds are laying eggs earlier. *Nature* **2**, 526.

Crozier, L. (2003). Winter warming facilitates range expansion: cold tolerance of the butterfly *Atalopedes campestris*. *Oecologia* **135**, 648–656.

Crutzen, P. J. (2002). Geology of mankind. *Nature* **415**, 23.

Czajka, M. C. and Lee, R. E. (1990). A rapid cold-hardening response protecting against cold shock injury in *Drosophila melanogaster*. *Journal of Experimental Biology* **148**, 245–254.

Danks, H. V (1987). *Insect dormancy: An ecological perspective*. Entomological Society of Canada, Ontario, Canada.

Danks, H. V (2002). The range of insect dormancy responses. *European Journal of Entomology* **99**, 127–142.

Danks, H. V (2006). Insect adaptations to cold and changing climate. *Canadian Entomology* **138**, 1–23.

Davidson, J. (1944). On the relationship between temperature and rate of development of insects at constant temperatures. *Journal of Animal Ecology* **13**, 26–38.

Davies, L. (1950). The hatching mechanism of Muscid eggs (Diptera). *Journal of Experimental Biology* **27**, 437–445.

De Kort, C. A. D. (1990). Thirty-five years of diapause research with the Colorado potato beetle. *Entomologica Experimentalis et Applicata* **56**, 1–13.

Denlinger, D. L. (1972). Induction and termination of pupal diapause in *Sarcophaga* (diptera: Sarcophagidae). *Biological Bulletin* **142**, 11–14.

Denlinger, D. L. (1991). Relationship between cold hardiness and diapause. In *Insects at Low Temperature* (ed. Lee, R.E. and Denlinger, D. L.), pp. 174 – 198. Chapman & Hall, New York, USA

Denlinger, D. L. (2002). Regulation of diapause. *Annual Review of Entomology* **47**, 93–122.

Denlinger, D. L. and Lee, R. E. (2010). *Low temperature biology of insects*. (ed. Denlinger, D. L. and Lee, J.).Cambridge University Press, Cambridge, UK.

Deutsch, C. A., Tewksbury, J. J., Huey, R. B., Sheldon, K. S., Ghalambor, C. K., Haak, D. C. and Martin, P. R. (2008). Impacts of climate warming on terrestrial ectotherms across latitude Thermal Safety margin. *Proceedings of the National Academy of Sciences of the United States of America* **105**, 6668–6672.

Dipterists Forum (2013). *Hoverfly recording scheme*. http://www.hoverfly.org.uk

Duman, J. G. (1982). Insect antifreezes and ice-nucleating agents. *Cryobiology* **19**, 613–627.

Duman, J. G. and Patterson, J. (1978). The role of ice nucleators in the frost tolerance of overwintering queens of the bald faced hornet. *Biochemistry and Physiology Part A: Physiology* **59**, 69–72.

Duman, J. G., Li, N., Verleye, D., Goetz, F. W., Wu, D. W., Andorfer, C. A., Benjamin, T. and Parmelee, D. C. (1998). Molecular characterization and sequencing of antifreeze proteins from larvae of the beetle *Dendroides canadensis*. *Journal of Comparative Physiology B: Biochemical, Systemic, and Environmental Physiology* **168**, 225–232.

Easterling, D. R., Meehl, G. A, Parmesan, C., Changnon, S. A, Karl, T. R. and Mearns, L. O. (2000). Climate extremes: observations, modeling, and impacts. *Science* **289**, 2068–2074.

Ehrlich, R., Miller, S. and Walker, R. L. (1970). Relationship between atmospheric temperature and survival of airborne bacteria. *Applied Microbiology* **19**, 245–249.

Eizaguirre, M., Lopez, C., Asin, L. and Albajes, R. (1994). Thermoperiodism, photoperiodism and sensitive stage in the diapause induction of *Sesamia nonagrioides* (Lepidoptera: Noctuidae). *Journal of Insect Physiology* **40**, 113 – 119.

Emelianov, I., Mallet, J. and Baltensweilert, W. (1995). Genetic differentiation in *Zeiraphera diniana* (Lepidoptera : Tortricidae, the larch budmoth): polymorphism , host races or sibling species? *Heredity* **75**, 416–424.

Emerson, K. J., Bradshaw, W. E. and Holzapfel, C. M. (2010). Microarrays reveal early transcriptional events during the termination of larval diapause in natural populations of the mosquito, *Wyeomyia smithii*. *PLoS ONE* **5**, e9574.

Everatt, M. J., Worland, M. R., Bale, J. S., Convey, P. and Hayward, S. A L. (2012). Pre-adapted to the maritime Antarctic? - Rapid cold hardening of the midge, *Eretmoptera murphyi*. *Journal of Insect Physiology* **58**, 1104–111.

Everatt, M. J., Bale, J. S., Convey, P., Worland, M. R. and Hayward, S. A L. (2013). The effect of acclimation temperature on thermal activity thresholds in polar terrestrial invertebrates. *Journal of Insect Physiology* **59**, 1057–1064.

Feder, M. E. and Hoffmann, G. E. (1999). Heat-shock proteins, molecular chaperones, and the stress response: evolutionary and ecological physiology. *Annual Review of Physiology* **61**, 243–282.

Fisher, R. (1980). The genetical theory of natural selection. Clarendon Press, Oxford, UK

Fischer, K. and Karl, I. (2010). Exploring plastic and genetic responses to temperature variation using copper butterflies. *Climate Research* **43**, 17–30.

Forrest, J. and Miller-Rushing, A. J. (2010). Toward a synthetic understanding of the role of phenology in ecology and evolution. *Philosophical Transactions of the Royal Society B: Biological Sciences* **365**, 3101–3112.

Franke, K., Dierks, A. and Fischer, K. (2012). Directional selection on cold tolerance does not constrain plastic capacity in a butterfly. *BMC Evolutionary Biology* **12**, 235.

Franks, S. J. and Hoffmann, A. A. (2012). Genetics of climate change adaptation. *Annual Review of Genetics* **46**, 185–208.

Gäde, G. and Auerswald, L. (2002). Beetles' choice - proline for energy output: control by AKHs. *Comparative Biochemistry and Physiology. Part B, Biochemistry & Molecular Biology* **132**, 117–129.

Gammie, S.C., Garland, T. and Stevenson, S.A. (2006). Artificial selection for increased maternal defence behaviour in mice. *Behavioural Genetics* **36**, 713–722.

Gibert, P. and Huey, R. B. (2011). Chill-coma temperature in *Drosophila*: effects of developmental temperature, latitude, and phylogeny. *Physiological and Biochemical Zoology:* **74**, 429–434.

Gibert, P., Huey, R. B. and Gilchrist, G. W. (2001). Locomotor performance of *Drosophila melanogaster*: interactions among developmental and adult temperatures, age, and geography. *Evolution***55**, 205–209.

Gilbert, F. S. (1984). Thermoregulation and the structure of swarms in *Syrphus ribesii* (Syrphidae). *OIKOS* **42**, 249–255.

Gilchrist, G.W. and Huey, R.B. (1998). The direct response of *Drosophila melanogaster* to selection on knockdown temperature. *Heredity* **83**, 15-29.

Gilkeson, L. A. and Hill, S. B. (1986). Genetic selection for an evaluation of nondiapause lines of the predatory midge, *Aphidoletes aphidimyza* (Rondani) (Diptera: Cecidomyiidae). *The Canadian Entomologist* **118**, 869–879.

Gomi, T., Nagasaka, M., Fukuda, T. and Hagihara, H. (2007). Shifting of the life cycle and life-history traits of the fall webworm in relation to climate change. *Entomologia Experimentalis et Applicata* **125**, 179–184.

Gordo, O. and Sanz, J. J. (2005). Phenology and climate change: a long-term study in a Mediterranean locality. *Oecologia* **146**, 484–495.

Goto, M., Sekine, Y., Outa, H., Hujikura, M. and Koichi, S. (2001). Relationships between cold hardiness and diapause, and between glycerol and free amino acid contents in overwintering larvae of the oriental corn borer, *Ostrinia furnacalis*. *Journal of Insect Physiology* **47**, 157–165.

Graham-Taylor, L. G., Stubbs, A. E. and Brooke, M. D. L. (2009). Changes in phenology of hoverflies in a central England garden. *Insect Conservation and Diversity* **2**, 29–35.

Grimaldi, D. and Engel, M. S. (2005). *Evolution of the Insects*. Cambridge University Press, Cambridge, UK.

Hahn, D. A. and Denlinger, D. L. (2007). Meeting the energetic demands of insect diapause: nutrient storage and utilization. *Journal of Insect Physiology* **53**, 760–773.

Hahn, D. A. and Denlinger, D. L. (2011). Energetics of insect diapause. *Annual Review of Entomology* **56**, 103–121.

Hanson, S. M. and Craig, G. B. (1994). Cold acclimation, diapause, and geographic origin affect cold hardiness in eggs of *Aedes albopictus* (Diptera: Culicidae). *Journal of Medical Entomology* **31**, 192–201.

Hart, A. J. and Bale, J.S. (1998). Factors affecting the freeze tolerance of the Hoverfly *Syrphus ribesii* (Diptera: Syrphidae). *Journal of Insect Physiology* **44**, 21–29.

Hart, A. J., Bale, J. S., Tullett, A. G., Worland, M. R. and Walters, K. F. (2002). Effects of temperature on the establishment potential of the predatory mite *Amblyseius californicus* McGregor (Acari: Phytoseiidae) in the UK. *Journal of Insect Physiology* **48**, 593–599.

Hassall, C., Thompson, D. J., French, G. C. and Harvey, I. F. (2007). Historical changes in the phenology of British Odonata are related to climate. *Global Change Biology* **13**, 933–941.

Hawes, T. C., Hines, A., Viant, M. R., Bale, J. S., Worland, M. R. and Convey, P. (2008). Metabolomic fingerprint of cryo-stress in a freeze tolerant insect. *Cryo Letters* **29**, 505–515.

Hayward, S. A. L., Pavlides, S. C., Tammariello, S. P., Rinehart, J. P. and Denlinger, D. L. (2005). Temporal expression patterns of diapause-associated genes in flesh fly pupae from the onset of diapause through post-diapause quiescence. *Journal of Insect Physiology* **51**, 631–640.

Hayward, S. A. L., Rinehart, J. P., Sandro, L. H., Lee, R. E. and Denlinger, D. L. (2007). Slow dehydration promotes desiccation and freeze tolerance in the Antarctic midge *Belgica antarctica*. *Journal of Experimental Biology* **210**, 836–844.

Hazell, S. P. and Bale, J. S. (2011). Low temperature thresholds: are chill coma and CT(min) synonymous? *Journal of Insect Physiology* **57**, 1085–1089.

Hazell, S. P., Pedersen, B. P., Worland, M. R., Blackburn, T. M. and Bale, J. S. (2008). A method for the rapid measurement of thermal tolerance traits in studies of small insects. *Physiological Entomology* **33**, 389–394.

Hazell, S. P., Groutides, C., Neve, B. P., Blackburn, T. M. and Bale, J. S. (2010). A comparison of low temperature tolerance traits between closely related aphids from the tropics, temperate zone, and Arctic. *Journal of Insect Physiology* **56**, 115–122.

Henrich, V. C. and Denlinger, D. L. (1982). Selection for late pupariation affects diapause incidence and duration in the flesh fly, *Sarcophaga bullata*. *Physiological Entomology* **7**, 407–411.

Henrich, V. C. and Denlinger D L. (1983). Genetic differences in pupal diapause incidence between two selected strains of the flesh fly. *Journal of Heredity* **74**, 371–374.

Hickling, R., Roy, D. B., Hill, J. K., Fox, R. and Thomas, C. D. (2006). The distributions of a wide range of taxonomic groups are expanding polewards. *Global Change Biology* **12**, 450–455.

Ho, D. H. and Burggren, W. W. (2010). Epigenetics and transgenerational transfer: a physiological perspective. *Journal of Experimental Biology* **213**, 3–16.

Hockham, L., Graves, J. and Ritchie, M. (2001). Variable maternal control of facultative egg diapause in the bushcricket *Ephippiger ephippiger*. *Ecological Entomology* **26**, 143–147.

Hodkova, M. and Hodek, I. (1994). Control of diapause and supercooling by the retrocerebral complex in *Pyrrhocoris apterus*. *Entomologia Experimentalis et Applicata* **70**, 237–245.

Hodkova, M. and Hodek, I. (1997). Temperature regulation of supercooling and gut nucleation in relation to diapause of *Pyrrhocoris apterus* (L.) (Heteroptera). *Cryobiology* **34**, 70–79.

Hodkova, M. and Hodek, I. (2004). Photoperiod, diapause and cold-hardiness. *European Journal of Entomology* **101**, 445–458.

Hoffmann, A. A. (2010). A genetic perspective on insect climate specialists. *Australian Journal of Entomology* **49**, 93–103.

Hoffmann, A. A. and Sgrò, C. M. (2011). Climate change and evolutionary adaptation. *Nature* **470**, 479–485.

Hoffmann, A. A., Anderson, A. and Hallas, R. (2002). Opposing clines for high and low temperature resistance in *Drosophila melanogaster*. *Ecology Letters* **5**, 614–618.

Hoffmann, A. A., Shirriffs, J. and Scott, M. (2005). Relative importance of plastic vs genetic factors in adaptive differentiation: geographical variation for stress resistance in *Drosophila melanogaster* from eastern Australia. *Functional Ecology* **19**, 222–227.

Hoffmann, A. A., Chown, S. L. and Clusella-Trullas, S. (2013). Upper thermal limits in terrestrial ectotherms: how constrained are they? *Functional Ecology* **27**, 934–949.

Holland, M. M. and Bitz, C. M. (2003). Polar amplification of climate change in coupled models. *Climate Dynamics* **21**, 221–232.

Horwarth, K. and Duman, J. G. (1983). Preparatory adaptations for winter survival in the cold hardy beetles, *Dendroides canadensis* and *Dendroides concolor*. *Journal of Comparative Physiology* **151**, 225–232.

Hou, M., Lin, W. and Han, Y. (2009). Seasonal changes in supercooling points and glycerol content in overwintering larvae of the Asiatic Rice Borer from rice and water-oat plants. *Environmental Entomology* **38**, 1182–1188.

Houten, Y. M. V, Stratum, P. V, Bruin, J. and Veerman, A. (1995). Selection for non-diapause in Amblyseius cucumeris and *Amblyseius barkeri* and exploration of the effectiveness of selected strains for thrips control. *Entomologia Experimentalis et Applicata* **77**, 289–295.

Howlett, B. G. (2012). Hybrid carrot seed crop pollination by the fly *Calliphora vicina* (Diptera: Calliphoridae). *Journal of Applied Entomology* **136**, 421–430.

Huber, J. T. and Beardsley, J. W. (2000). A New Genus of Fairyfly, *Kikiki*, from the Hawaiian Islands (Hymenoptera: Mymaridae). *Proceedings of the Hawaiian Entomological Society* **34**, 65–70.

Huey, R. B. and Stevenson, R. D. (1979). Integrating thermal physiology and ecology of ectotherms: A discussion of approaches. *Integrative and Comparative Biology* **19**, 357–366.

Huey, R. B., Berrigan, D., Gilchrist, G. W. and Herron, J. C. (1999). Testing the adaptive significance of acclimation: A strong inference approach. *American Zoologist* **39**, 323–336.

Hughes, G. E., Bale, J. S. and Sterk, G. (2009). Thermal biology and establishment potential in temperate climates of the predatory mirid *Nesidiocoris tenuis*. *BioControl* **54**, 785–795.

Hughes, G. E., Alford, L., Sterk, G. and Bale, J. S. (2010). Thermal activity thresholds of the predatory mirid *Nesidiocoris tenuis*: implications for its efficacy as a biological control agent. *BioControl* **55**, 493–501.

Hutchison, V. H. and Maness, J. D. (1979). The role of behavior in temperature acclimation and tolerance in ectotherms. *Integrative and Comparative Biology* **19**, 367–384.

Hwang, C. and Turner, B. D. (2005). Spatial and temporal variability of necrophagous Diptera from urban to rural areas. *Medical and Veterinary Entomology* **19**, 379–391.

Ikeno, T., Tanaka, S. I., Numata, H. and Goto, S. G. (2010). Photoperiodic diapause under the control of circadian clock genes in an insect. *BMC Biology* **8**, 116.

IPCC (2013). Twelfth Session of Working Group 1, Summary for Policymakers. In (ed. Alexander, L., Allen, S., Bindoff, N. L., Breon, F.-M., Church, J., and Al, E.), pp. 1–36. Cambridge University Press, Cambridge, UK.

Jaiser, R., Dethloff, K., Handorf, D., Rinke, A. and Cohen, J. (2012). Impact of sea ice cover changes on the Northern Hemisphere atmospheric winter circulation. *Tellus A* **64**, 1–11.

Jepsen, J. U., Kapari, L., Hagen, S. B., Schott, T., Vindstad, O. P. L., Nilssen, A. C. and Ims, R. A. (2011). Rapid northwards expansion of a forest insect pest attributed to spring phenology matching with sub-Arctic birch. *Global Change Biology* **17**, 2071–2083.

Jobling, M. (1981). Temperature tolerance and the final preferendum-rapid methods for the assessment of optimum growth temperatures. *Journal of Fish Biology* **19**, 439–455.

Johnson, B. (2013). Effect of stress and diapause in two Calliphoridae species. Unpublished PhD thesis, University of Birmingham, Birmingham, UK

Ju, R.T., Xiao, Y.Y. and Li, B. (2011). Rapid cold hardening increases cold and chilling tolerances more than acclimation in the adults of the sycamore lace bug, *Corythucha ciliata* (Say) (Hemiptera: Tingidae). *Journal of Insect Physiology* **57**, 1577–1582.

Kalushkov, O. and Nedved, P. (2000). Cold hardiness of *Pyrrhocoris apterus* (Heteroptera: Pyrrhocoridae) from central and southern Europe. *European Journal of Entomology* **97**, 149–153.

Kaneshrajah, G. and Turner, B. (2004). *Calliphora vicina* larvae grow at different rates on different body tissues. *International Journal of Legal Medicine* **118**, 242–244.

Kappus, K. D. and Venard, C. E. (1967). The effects of photoperiod and temperature on the induction of diapause in *Aedes triseriatus* (Say). *Journal of Insect Physiology* **13**, 1007–1019.

Kelty, J. (2007). Rapid cold-hardening of *Drosophila melanogaster* in a field setting. *Physiological Entomology* **32**, 343–350.

Kelty, J. D. and Lee, R. E. (1999). Induction of rapid cold hardening by cooling at ecologically relevant rates in *Drosophila melanogaster*. *Journal of insect physiology* **45**, 719–726.

Kelty, J. D. and Lee, R. E. (2001). Rapid cold-hardening of *Drosophila melanogaster* (Diptera: Drosophilidae) during ecologically based thermoperiodic cycles. *Journal of Experimental Biology* **1666**, 1659–1666.

Khodayari, S., Moharramipour, S., Larvor, V., Hidalgo, K. and Renault, D. (2013). Deciphering the metabolic changes associated with diapause syndrome and cold acclimation in the two-spotted spider mite *Tetranychus urticae*. *PLoS ONE* **8**, e54025.

Kodrík, D., Socha, R. and Šula, J. (1995). Hexameric haemolymph protein related to adult diapause in the red firebug, *Pyrrhocoris apterus* (L.) (Heteroptera). *Journal of Insect Physiology* **41**, 793–800.

Kohshima, S. (1984). A novel cold-tolerant insect found in a Himalayan glacier. *Nature* **310**, 225–227.

Kostál, V., Zahradnícková, H., Šimek, P., Koštál, V. and Simek, P. (2011). Hyperprolinemic larvae of the drosophilid fly, *Chymomyza costata*, survive cryopreservation in liquid nitrogen. *Proceedings of the National Academy of Sciences of the United States of America* **108**, 13041–13046.

Koštál, V. (2006). Eco-physiological phases of insect diapause. *Journal of Insect Physiology* **52**, 113–127.

Koštál, V. (2011). Insect photoperiodic calendar and circadian clock: independence, cooperation, or unity? *Journal of Insect Physiology* **57**, 538–556.

Koštál, V. and Simek, P. (2000). Overwintering strategy in *Pyrrhocoris apterus* (Heteroptera): the relations between life-cycle, chill tolerance and physiological adjustments. *Journal of Insect Physiology* **46**, 1321–1329.

Koštál, V., Simůnková, P., Kobelková, A., Shimada, K., Simunkova, P. and Kobelkova, A. (2009). Cell cycle arrest as a hallmark of insect diapause: Changes in gene transcription during diapause induction in the drosophilid fly, *Chymomyza costata*. *Insect Biochemistry and Molecular Biology* **39**, 875–883.

Koštál, V., Korbelová, J., Rozsypal, J., Zahradnicková, H., Cimlová, J., Tomčala, A. and Šimek, P. (2011a). Long-term cold acclimation extends survival time at 0°C and modifies the metabolomic profiles of the larvae of the fruit fly *Drosophila melanogaster*. *PLoS ONE* **6**, e25025.

Koštál, V., Doležal, P., Rozsypal, J., Moravcová, M., Zahradníčková, H. and Simek, P. (2011b). Physiological and biochemical analysis of overwintering and cold tolerance in two Central European populations of the spruce bark beetle, *Ips typographus*. *Journal of Insect Physiology* **57**, 1136–1146.

Koštál, V., Renault, D. and Rozsypal, J. (2011c). Seasonal changes of free amino acids and thermal hysteresis in overwintering heteropteran insect, *Pyrrhocoris apterus*. *Comparative Biochemistry and Physiology. Part A, Molecular & Integrative Physiology* **160**, 245–251.

Koštál, V., Šimek, P., Zahradníčková, H., Cimlová, J. and Štětina, T. (2012). Conversion of the chill susceptible fruit fly larva (*Drosophila melanogaster*) to a freeze tolerant organism. *Proceedings of the National Academy of Sciences* **109**, 3270–3274.

Koveos, D. S. (2001). Rapid cold hardening in the olive fruit fly *Bactrocera oleae* under laboratory and field conditions. *Entomologia Experimentalis et Applicata* **101**, 257–263.

Kristensen, T. N., Hoffmann, A. A., Overgaard, J., Sørensen, J. G., Hallas, R. and Loeschcke, V. (2008). Costs and benefits of cold acclimation in field-released Drosophila. *Proceedings of the National Academy of Sciences* **105**, 216–221.

Kurban, A., Yoshida, H., Izumi, Y., Sonoda, S. and Tsumuki, H. (2005). Pupal diapause of *Helicoverpa armigera*: sensitive stage for photoperiodic induction. *Applied Entomology and Zoology* **40**, 457–460.

Kyriacou, C. P., Peixoto, A. A, Sandrelli, F., Costa, R. and Tauber, E. (2008). Clines in clock genes: fine-tuning circadian rhythms to the environment. *Trends in Genetics* **24**, 124–32.

Labandeira, C. and Sepkoski, J. (1993). Insect Diversity in the Fossil Record. *Science* **261**, 310–315.

Ladanyi, M. and Horvath, L. (2010). A review of the potential climte change impact on insect populations; general and agricultural aspects. *Applied Ecology and Environmental Research* **8**, 143–152.

Lalouette, L., Koštál, V., Colinet, H., Gagneul, D. and Renault, D. (2007). Cold exposure and associated metabolic changes in adult tropical beetles exposed to fluctuating thermal regimes. *The FEBS Journal* **274**, 1759–1767.

Larsen, K. and Lee, R. J. (1994). Cold tolerance including rapid cold-hardening and inoculative freezing of fall migrant monarch butterflies in Ohio. *Journal of Insect Physiology* **40**, 859–864.

Lebouvier, M., Laparie, M., Hullé, M., Marais, A., Cozic, Y., Lalouette, L., Vernon, P., Candresse, T., Frenot, Y. and Renault, D. (2011). The significance of the sub-Antarctic Kerguelen Islands for the assessment of the vulnerability of native communities to climate change, alien insect invasions and plant viruses. *Biological Invasions* **13**, 1195–1208.

Lee, R. E. and Baust, J. G. (1987). Multiple stress tolerance in an antarctic terrestrial arthropod: *Belgica antarctica*. *Cryobiology* **24**, 140–147.

Lee, R. E. and Denlinger, D. L. (1985). Cold tolerance in diapausing and non-diapausing stages of the flesh fly, *Sarcophaga crassipalpis*. *Physiological Entomology* **10**, 309–315.

Lee, R. E. and Denlinger, D. L. (1991). Insects at low temperatures. Cambridge university press, Cambridge, UK

Lee, R. E., Chen, C. P. and Denlinger, D. L. (1987). A rapid cold-hardening process in insects. *Science* **238**, 1415–1417.

Lee, R. J., Costanzo, J. P. and Mugnano, J. A. (1996). Regulation of supercooling and ice nucleation in insects. *European Journal of Entomology* **93**, 405–418.

Lee, R. E., Elnitsky, M. A, Rinehart, J. P., Hayward, S. A L., Sandro, L. H. and Denlinger, D. L. (2006). Rapid cold-hardening increases the freezing tolerance of the Antarctic midge Belgica antarctica. *Journal of Experimental Biology* **209**, 399–406.

Lee, R. E., Damodaran, K., Yi, S.-X. and Lorigan, G. A (2006a). Rapid cold-hardening increases membrane fluidity and cold tolerance of insect cells. *Cryobiology* **52**, 459–463.

Lees, A. D. (1955). *The Physiology of Diapause in Arthropods*. CUP Archive, New York. New York, USA.

Li, Y.-P., Goto, M., Ito, S., Sato, Y., Sasaki, K. and Goto, N. (2001). Physiology of diapause and cold hardiness in the overwintering pupae of the fall webworm *Hyphantria cunea* (Lepidoptera: Arctiidae) in Japan. *Journal of Insect Physiology* **47**, 1181–1187.

Losey, J. E. and Vaughan, M. (2006). The economic value of ecological services provided by insects. *BioScience* **56**, 311.

Lynch, C. (1980). Responses to divergent selection for nest building in *Mus musculus*. *Genetics* **96**, 757–765.

Lynch, C. and Walsh, B. (1998). Genetics and analysis of quantative traits. Sinauer Associates, Connecticut, USA.

MacDonald, G. M. (2010). Global warming and the Arctic: a new world beyond the reach of the Grinnellian niche? *Journal of Experimental Biology* **213**, 855–861.

Macdonald, S. S., Rako, L., Batterham, P. and Hoffmann, A. A (2004). Dissecting chill coma recovery as a measure of cold resistance: evidence for a biphasic response in *Drosophila melanogaster*. *Journal of Insect Physiology* **50**, 695–700.

Macmillan, H. A and Sinclair, B. J. (2011). Mechanisms underlying insect chill-coma. *Journal of Insect Physiology* **57**, 12–20.

Manachini, B., Billeci, N. and Palla, F. (2013). Exotic insect pests: The impact of the Red Palm Weevil on natural and cultural heritage in Palermo (Italy). *Journal of Cultural Heritage* **14**, e177–e182.

Manrique, V., Diaz, R. and Overholt, W. A. (2012). Temperature-dependent development, diapause and cold tolerance of *Gratiana graminea*, a potential biological control agent of Solanum viarum in Florida, USA. *BioControl* **57**, 581–589.

Mansingh, A. (1974). Studies on insect dormancy. II. Relationship of cold-hardiness to diapause and quiescence in the eastern tent caterpillar, *Malacosoma americanum* (Fab.), (Lasiocampidae: Lepidoptera). *Canadian Journal of Zoology* **52**, 629–637.

Mansingh, A. and Smallman, B. N. (1966). Photoperiod Control of an 'Obligatory' Pupal Diapause. *The Canadian Entomologist* **98**, 613–616.

Márquez, E. J., Rada, F. and Fariñas, M. R. (2006). Freezing tolerance in grasses along an altitudinal gradient in the Venezuelan Andes. *Oecologia* **150**, 393–397.

Marshall, D. J. (2008). Transgenerational plasticity in the sea: Context-dependent maternal effects across the life history. *Ecology* **89**, 418–427.

Marshall, K. E. and Sinclair, B. J. (2010). Repeated stress exposure results in a survival-reproduction trade-off in *Drosophila melanogaster*. *Proceedings of the National Academy of Sciences of the United States of America* **277**, 963–969.

Mayhew, P. J. (2007). Why are there so many insect species? Perspectives from fossils and phylogenies. *Biological Reviews of the Cambridge Philosophical Society* **82**, 425–454.

McWatters, H. G. and Saunders, D. S. (1996). The influence of each parent and geographic origin on larval diapause in the blow fly, *Calliphora vicina*. *Journal of Insect Physiology* **42**, 721–726.

McWatters, H. G. and Saunders, D. S. (1998). Maternal temperature has different effects on the photoperiodic response and duration of larval diapause in blow fly (*Calliphora vicina*) strains collected at two latitudes. *Physiological Entomology* **23**, 369–375.

Mellanby, K. (1939). Low temperature and insect activity. *Proceedings of the Royal Society B: Biological Sciences* **127**, 473–487.

Mercader, R. J. and Scriber, J. M. (2008). Asymmetrical thermal constraints on the parapatric species boundaries of two widespread generalist butterflies. *Ecological Entomology* **33**, 537–545.

Michaud, M. R. and Denlinger, D. L. (2006). Oleic acid is elevated in cell membranes during rapid cold-hardening and pupal diapause in the flesh fly, *Sarcophaga crassipalpis*. *Journal of Insect Physiology* **52**, 1073–1082.

Michaud, M. R. and Denlinger, D. L. (2007). Shifts in the carbohydrate, polyol, and amino acid pools during rapid cold-hardening and diapause-associated cold-hardening in flesh flies (*Sarcophaga crassipalpis*): a metabolomic comparison. *Journal of Comparative Physiology. B: Biochemical, Systemic, and Environmental Physiology* **177**, 753–763.

Michaud, M. R., Benoit, J. B., Lopez-Martinez, G., Elnitsky, M. a, Lee, R. E. and Denlinger, D. L. (2008). Metabolomics reveals unique and shared metabolic changes in response to heat shock, freezing and desiccation in the Antarctic midge, *Belgica antarctica*. *Journal of Insect Physiology* **54**, 645–655.

Milonas, P. G. and Savopoulou-Soultani, M. (2000). Temperature dependent development of the parasitoid *Colpoclypeus florus* (Hymenoptera: Eulophidae) in the laboratory. *Journal of Economic Entomology* **93**, 1627–1632.

Mondor, E. B., Tremblay, M. N. and Lindroth, R. L. (2004). Transgenerational phenotypic plasticity under future atmospheric conditions. *Ecology Letters* **7**, 941–946.

Morey, A. C., Hutchison, W. D., Venette, R. C. and Burkness, E. C. (2012). Cold Hardiness of *Helicoverpa zea* (Lepidoptera: Noctuidae) Pupae. *Environmental Entomology* **41**, 172–179.

Morrow, E.H. and Gage, M.J.G. (2001). Artificial selection and heritability of sperm length in *Gryllus bimaculatus*. *Heredity* **87**, 356–362.

Mousseau, T. A and Dingle, H. (1991). Maternal effects in insect life histories. *Annual Review of Entomology* **36**, 511–534.

Mousseau, T. A. and Fox, C. W. (1998). The adaptive significance of maternal effects. *Trends in Ecology and Evolution* **13**, 403–407.

Mousseau, T. A and Roff, D. (1987). Natural selection and the heritability of fitness components. *Heredity* **59**, 181–197.

Murata, Y., Novković, B., Suwito, A. and Kimura, M. T. (2013). Diapause and cold tolerance in Asian species of the parasitoid Leptopilina (Hymenoptera: Figitidae). *Physiological Entomology* **38**, 211–218.

Musolin, D. L. (2007). Insects in a warmer world: ecological, physiological and life-history responses of true bugs (Heteroptera) to climate change. *Global Change Biology* **13**, 1565–1585.

Musolin, D. L. (2012). Surviving winter: diapause syndrome in the southern green stink bug *Nezara viridula* in the laboratory, in the field, and under climate change conditions. *Physiological Entomology* **37**, 309–322.

Musolin, D. L. and Numata, H. (2004). Late-season induction of diapause in *Nezara viridula* and its effect on adult coloration and post-diapause reproductive performance. *Entomologia Experimentalis et Applicata* **111**, 1–6.

Musolin, D. L., Tougou, D. and Fujisaki, K. (2010). Too hot to handle? Phenological and life-history responses to simulated climate change of the southern green stink bug *Nezara viridula* (Heteroptera: Pentatomidae). *Global Change Biology* **16**, 73–87.

Musselman, L. P., Fink, J. L., Narzinski, K., Ramachandran, P. V., Hathiramani, S. S., Cagan, R. L. and Baranski, T. J. (2011). A high-sugar diet produces obesity and insulin resistance in wild-type *Drosophila*. *Journal of Cell Science* **124**, e1.

Nice, C. C. and Fordyce, J. A. (2006). How Caterpillars Avoid Overheating: Behavioral and Phenotypic Plasticity of Pipevine Swallowtail Larvae. *Oceologia* **146**, 541–548.

NOAA (2013). http://www.esrl.noaa.gov/gmd/ccgg/trends/.

Numata, H., Shiga, S. and Morita, A. (1997). Photoperiod receptors in Arthropods. *Zoological Science* **32**, 187–197.

Nunamaker, R. A. (1993). Rapid coldhHardening in *Culicoides variipennis sonorensis* (Diptera: Ceratopogonidae). *Journal of Medical Entomology* **30**, 5–7.

Nunes, M. V. and Saunders, D. (1999). Photoperiodic time measurement in insects: A review of clock models. *Journal of Biological Rhythms* **14**, 84–104.

Nyamukondiwa, C. and Terblanche, J. (2010). Rapid cold-hardening in *Zaprionus vittiger* (Coquillett) (Diptera: Drosophilidae). *Cryo Letters* **31**, 504–512.

Oliveira, C. M., Auad, A. M., Mendes, S. M. and Frizzas, M. R. (2013). Economic impact of exotic insect pests in Brazilian agriculture. *Journal of Applied Entomology* **137**, 1–15.

Otaki, J. M., Hiyama, A., Iwata, M. and Kudo, T. (2010). Phenotypic plasticity in the range-margin population of the lycaenid butterfly *Zizeeria maha*. *BMC Evolutionary Biology* **10**, 252.

Overgaard, J., Malmendal, A., Sørensen, J. G., Bundy, J. G., Loeschcke, V., Nielsen, N. C. and Holmstrup, M. (2007). Metabolomic profiling of rapid cold hardening and cold shock in *Drosophila melanogaster*. *Journal of Insect Physiology* **53**, 1218–1232.

Owen, E. L., Bale, J. S. and Hayward, S. A L. (2013). Can winteraActive bumblebees survive the cold? Assessing the cold tolerance of *Bombus terrestris audax* and the effects of pollen feeding. *PLoS ONE* **8**, e80061.

Parmesan, C. (2006). Ecological and evolutionary responses to recent climate change. *Annual Review of Ecology, Evolution, and Systematics* **37**, 637–669.

Parmesan, C. and Yohe, G. (2003). A globally coherent fingerprint of climate change impacts across natural systems. *Nature* **421**, 37–42.

Parsons, M. (1986). *Threatened Swalowtail Butterflies of the World: The IUCN Red Data Book*. IUCN, Gland, Switzerland and Cambridge, UK.

Peck, L. S., Clark, M. S., Morley, S. A., Massey, A. and Rossetti, H. (2009). Animal temperature limits and ecological relevance: effects of size, activity and rates of change. *Functional Ecology* **23**, 248–256.

Penuelas, J. and Filella, I. (2001). Responses to a Warming World. *Science* **294**, 64–65.

Penuelas, J., Filella, I. and Comas, P. (2002). Changed plant and animal life cycles from 1952 to 2000 in the Mediterranean region. *Global Change Biology* **8**, 531–544.

Peñuelas, J., Sardans, J., Estiarte, M., Ogaya, R., Carnicer, J., Coll, M., Barbeta, A., Rivas-Ubach, A., Llusià, J., Garbulsky, M. (2013). Evidence of current impact of climate change on life: a walk from genes to the biosphere. *Global Change Biology* **10,** 2303–2338.

Philip, B. N., Yi, S.-X., Elnitsky, M. A. and Lee, R. E. (2008). Aquaporins play a role in desiccation and freeze tolerance in larvae of the goldenrod gall fly, *Eurosta solidaginis*. *Journal of Experimental Biology* **211**, 1114–1119.

Plautz, S. C., Guest, T., Funkhouser, M. A. and Salice, C. J. (2013). Transgenerational cross-tolerance to stress : parental exposure to predators increases offspring contaminant tolerance. *Ecotoxicology* **22**, 854–861.

Pörtner, H. O. (2002). Climate variations and the physiological basis of temperature dependent biogeography: systemic to molecular hierarchy of thermal tolerance in animals. *Comparative Biochemistry and Physiology. Part A, Molecular & Integrative Physiology* **132**, 739–761.

Potts, S. G., Biesmeijer, J. C., Kremen, C., Neumann, P., Schweiger, O. and Kunin, W. E. (2010). Global pollinator declines: trends, impacts and drivers. *Trends in Ecology & Evolution* **25**, 345–353.

Powell, J. A. (1992). Interrelationships of yuccas and yucca moths. *Trends in Ecology & Evolution* **7**, 10–15.

Powell, S. J. and Bale, J. S. (2004). Cold shock injury and ecological costs of rapid cold hardening in the grain aphid *Sitobion avenae* (Hemiptera: Aphididae). *Journal of Insect Physiology* **50**, 277–384.

Powell, S. J. and Bale, J. S. (2006). Effect of long-term and rapid cold hardening on the cold torpor temperature of an aphid. *Physiological Entomology* **31**, 348–352.

Pullin, A. (1996). Physiological relationships between insect diapause and cold tolerance: Coevolution or coincidence? *European Journal of Entomology* **93**, 121–129.

Pullin, A. and Wolda, H. (1993). Glycerol and glucose accumulation during diapause in a tropical beetle. *Physiological Entomology* **18**, 75–78.

Pullin, A., Bale, J. S. and Fontaine, X. L. R. (1991). Physiological aspects of diapause and cold tolerance in *Pieris brassicae*. *Physiological Entomology* **16**, 456–477.

Rabeling, C., Brown, J. M. and Verhaagh, M. (2008). Newly discovered sister lineage sheds light on early ant evolution. *Proceedings of the National Academy of Sciences of the United States of America* **105**, 14913–14917.

Radchuk, V., Turlure, C. and Schtickzelle, N. (2013). Each life stage matters: the importance of assessing the response to climate change over the complete life cycle in butterflies. *The Journal of Animal Ecology* **82**, 275–285.

Ragland, G. J., Denlinger, D. L. and Hahn, D. A (2010). Mechanisms of suspended animation are revealed by transcript profiling of diapause in the flesh fly. *Proceedings of the National Academy of Sciences of the United States of America* **107**, 14909–14914.

Rako, L. and Hoffmann, A. A (2006). Complexity of the cold acclimation response in *Drosophila melanogaster*. *Journal of Insect Physiology* **52**, 94–104.

Ramløv, H. (2000). Aspects of natural cold tolerance in ectothermic animals. *Human Reproduction* **15** , 26–46.

Rando, O. J. and Verstrepen, K. J. (2007). Timescales of genetic and epigenetic inheritance. *Cell* **128**, 655–668.

Réale, D., McAdam, A. G., Boutin, S. and Berteaux, D. (2003). Genetic and plastic responses of a northern mammal to climate change. *Proceedings of the National Academy of Sciences* **270**, 591–6.

Reinders, J., Wulff, B. B. H., Mirouze, M., Marí-Ordóñez, A., Dapp, M., Rozhon, W., Bucher, E., Theiler, G. and Paszkowski, J. (2009). Compromised stability of DNA methylation and transposon immobilization in mosaic *Arabidopsis epigenomes*. *Genes & Development* **23**, 939–950.

Renault, D., Salin, C., Vannier, G. and Vernon, P. (1999). Survival and chill-coma in the adult lesser mealworm, *Alphitobius diaperinus* (Coleoptera: Tenebrionidae), exposed to low temperatures. *Journal of Thermal Biology* **24**, 229–236.

Renault, D., Salin, C., Vannier, G. and Vernon, P. (2002). Survival at low temperatures in insects: what is the ecological significance of the supercooling point? *Cryo Letters* **23**, 217–218.

Richard, D. and Saunders, D. S. (1987). Prothoracic gland function in diapause and non-diapause *Sarcopha gaargyrostoma* and *Calliphora vicina*. *Journal of Insect Physiology* **33**, 385–392.

Richter, O., Suhling, F., Müller, O. and Kern, D. (2008). A model for predicting the emergence of dragonflies in a changing climate. *Freshwater Biology* **53**, 1868–1880.

Rinehart, J. P., Yocum, G. D. and Denlinger, D. L. (2000). Thermotolerance and rapid cold hardening ameliorate the negative effects of brief exposures to high or low temperatures on fecundity in the flesh fly, *Sarcophaga crassipalpis*. *Physiological Entomology* **25**, 330–336.

Rinehart, J. P., Li, A., Yocum, G. D., Robich, R. M., Hayward, S. A. L. and Denlinger, D. L. (2007). Up-regulation of heat shock proteins is essential for cold survival during insect diapause. *Proceedings of the National Academy of Sciences of the United States of America* **104**, 11130–11137.

Ring, R. A. (1972). Relationship between diapause and supercooling in the blowfly, *Lucilia sericata* (Mg.) (Diptera: Calliphoridae). *Canadian Journal of Zoology* **50**, 1601–1605.

Ring, R. A. (1982). Freezing tolerant insects with low supercooling points. *Comparative Biochemistry and Physiology* **73**, 605–612.

Ring, R. A. and Tesar, D. (1981). Adaptations to cold in Canadian Arctic insects. *Cryobiology* **18**, 199–211.

Rivers, D. B., Lee, R. E. and Denlinger, D. L. (2000). Cold hardiness of the fly pupal parasitoid *Nasonia vitripennis* is enhanced by its host *Sarcophaga crassipalpis*. *Journal of Insect Physiology* **46**, 99–106.

Roy, D. B. and Sparks, T. H. (2000). Phenology of British butterflies and climate change. *Global Change Biology* **6**, 407–416.

Rozsypal, J., Koštál, V., Zahradníčková, H. and Šimek, P. (2013). Overwintering strategy and mechanisms of cold tolerance in the codling moth (*Cydia pomonella*). *PLoS ONE* **8**, e61745.

Ruddiman, W. F. and Thomson, J. S. (2001). The case for human causes of increased atmospheric CH 4 over the last 5000 years. *Science* **20**, 1769–1777.

Salinas, S. and Munch, S. B. (2012). Thermal legacies: transgenerational effects of temperature on growth in a vertebrate. *Ecology Letters* **15**, 159–163.

Salminen, T. S. and Hoikkala, A. (2013). Effect of temperature on the duration of sensitive period and on the number of photoperiodic cycles required for the induction of reproductive diapause in *Drosophila montana*. *Journal of Insect Physiology* **59**, 450–457.

Salt, R. W. (1961). Principles of insect cold-hardiness. *Annual Review of Entomology* **6**, 55–74.

Saunders, D. S. (1972). Circadian control of larval growth rate in *Sarcophaga argyrostoma*. *Proceedings of the National Academy of Sciences of the United States of America* **69**, 2738–2740.

Saunders, D. S. (1987). Maternal influence on the incidence and duration of larval diapause in *Calliphora vicina*. *Physiological Entomology* **12**, 331–338.

Saunders, D. (1997a). Insect circadian rhythms and photoperiodism. *Invertebrate Neuroscience* **3**, 155–164.

Saunders, D. S. (1997b). Under-sized larvae from short-day adults of the blow fly. *Calliphora vicina*, side-step the diapause programme. *Physiological Entomology* **22**, 249–255.

Saunders, D. S. (2013). Insect photoperiodism: measuring the night. *Journal of Insect Physiology* **59**, 1–10.

Saunders, D. S. and Cymborowski, B. (2003). Selection for high diapause incidence in blow flies (*Calliphora vicina*) maintained under long days increases the maternal critical daylength: some consequences for the photoperiodic clock. *Journal of Insect Physiology* **49**, 777–784.

Saunders, D. S. and Hayward, S. A. L. (1998). Geographical and diapause-related cold tolerance in the blow fly, *Calliphora vicina*. *Journal of Insect Physiology* **44**, 541–551.

Saunders, D. S., Cairncross KD and Macpherson J N (1986). Maternal and larval effects of photoperiod on the induction of larval diapause in two species of fly, *Calliphora vicina* and *Lucilia sericata*. *Journal of Experimental Biology* **46**, 51–58.

Saunders, D. S., Henrich, V. C. and Gilbert, L. I. (1989). Induction of diapause in *Drosophila melanogaster*: photoperiodic regulation and the impact of arrhythmic clock mutations on time measurement. *Proceedings of the National Academy of Sciences of the United States of America* **86**, 3748–3752.

Schmidt, P. S., Matzkin, L., Ippolito, M. and Eanes, W. F. (2005). Geographic variation in diapause incidence, life-history traits, and climatic adaptation in *Drosophila melanogaster*. *Evolution* **59**, 1721–1732.

Schneiderman, H. A. and Horwitz, J. (1958). The induction and termination of facultative diapause in the chalcid wasps *Mormoniella vitripennis* (Walker) and *Tritneptis klugii* (Ratzeburg). *Journal of Experimental Biology* **35**, 520–551.

Sgolastra, F., Kemp, W. P., Buckner, J. S., Pitts-Singer, T. L., Maini, S. and Bosch, J. (2011). The long summer: Pre-wintering temperatures affect metabolic expenditure and winter survival in a solitary bee. *Journal of Insect Physiology* **57**, 1651–1659.

Sgolastra, F., Kemp, W. P., Maini, S. and Bosch, J. (2012). Duration of prepupal summer dormancy regulates synchronization of adult diapause with winter temperatures in bees of the genus *Osmia*. *Journal of Insect Physiology* **58**, 924–933.

Shaw, A. P. M., Torr, S. J., Waiswa, C., Cecchi, G., Wint, G. R. W., Mattioli, R. C. and Robinson, T. P. (2013). Estimating the costs of tsetse control options: an example for Uganda. *Preventive Veterinary Medicine* **110**, 290–303.

Shreve, S. M. (2004). Preservation of reproductive behaviors during modest cooling: rapid cold-hardening fine-tunes organismal response. *Journal of Experimental Biology* **207**, 1797–1802.

Simons, A. and Johnston, M. (1997). Developmental instability as a bet-hedging strategy. *Oikos* **80**, 401–406.

Sinclair, B. J. (1999). Insect cold tolerance: How many types of frozen? *European Journal of Entomology* **96**, 157–164.

Sinclair, B. J. and Chown, S. L. (2003). Rapid responses to high temperature and desiccation but not to low temperature in the freeze tolerant sub-Antarctic caterpillar *Pringleophaga marioni* (Lepidoptera, Tineidae). *Journal of Insect Physiology* **49**, 45–52.

Sinclair, B. J. and Chown, S. L. (2006). Rapid cold-hardening in a Karoo beetle, *Afrinus sp*. *Physiological Entomology* **31**, 98–101.

Sinclair, B. J., Vernon, P., Jaco Klok, C. and Chown, S. L. (2003). Insects at low temperatures: an ecological perspective. *Trends in Ecology & Evolution* **18**, 257–262.

Slachta, M., Berkova, P., Vambera, J. and Kostal, V. (2002). Physiology of cold-acclimation in non-diapausing adults of *Pyrrhocoris apterus* (Heteroptera). *European Journal of Entomology* **99**, 181–187.

Sobek-Swant, S., Crosthwaite, J. C., Lyons, D. B. and Sinclair, B. J. (2011). Could phenotypic plasticity limit an invasive species? Incomplete reversibility of mid-winter deacclimation in an emerald ash borer. *Biological Invasions* 115–125.

Solomon, S., Manning, D., Marquis, M., Averyt, K., Tignor, M., Miller. HL and Chen, Z. (2007). Regional Climate Projections. In climate change *2007: The physical science basis* (ed. Christensen, J. H. and Hewitson, B.), pp. 847–940. Cambridge University Press, Cambridge, UK.

Somme, L. (1999). The physiology of cold hardiness in terrestrial arthropods. *European Journal of Entomology* **96**, 1–10.

Sparks, T. H. and Carey, P. D. (1995). The responses of species to climate over two centuries : an analysis of the Marsham phenological record , 1736-1947. *Journal of Ecology* **83**, 321–329.

Storey, K. B. (1997). Organic solutes in freezing tolerance. *Comparative Biochemistry and Physiology Part A Physiology* **117**, 319–326.

Storey, J. M. and Storey, K. B. (1985). Triggering of cryoprotectant synthesis by the initiation of ice nucleation in the freeze tolerant frog, *Rana sylvatica*. *Journal of Comparative Physiology B* **156**, 191–195.

Storey, K. B. and Storey, J. M. (1992). Natural freeze tolerance in ectothermic vertebrates. *Annual Review of Physiology* **54**, 619–637.

Stork, N. E. (2009). Re-assessing current extinction rates. *Biodiversity and Conservation* **19**, 357–371.

Sunday, J. M., Bates, A. E. and Dulvy, N. K. (2011). Global analysis of thermal tolerance and latitude in ectotherms. *Proceedings of the National Academy of Sciences of the United States of America* **278**, 1823–30.

Suter, L. and Widmer, A. (2013). Environmental heat and salt stress induce transgenerational phenotypic changes in *Arabidopsis thaliana*. *PLoS ONE* **8**, 1–11.

Swallow, J.G., Carter, P.A. and Garland, T. (1998). Artificial selection for wheel running behaviour in house mouse. *Behaviour Genetics* **28**, 227–237.

Sweeney, B. W. and Vannote, R. L. (1982). Population Synchrony in Mayflies: A predator satiation hypothesis. *Evolution* **36**, 810–821.

Tachibana, S.-I. and Numata, H. (2004a). Effects of temperature and photoperiod on the termination of larval diapause in *Lucilia sericata* (Diptera: Calliphoridae). *Zoological Science* **21**, 197–202.

Tachibana, S.-I. and Numata, H. (2004b). Parental and direct effects of photoperiod and temperature on the induction of larval diapause in the blow fly *Lucilia sericata*. *Physiological Entomology* **29**, 39–44.

Tachibana, S., Numata, H. and Meigen, L. (2004). Maternal induction of larval diapause and its sensitive stage in the blow fly *Lucilia sericata* (Meigen) (Diptera : Calliphoridae). *Entomological Science* **7**, 231–235.

Tachibana, S.-I., Numata, H. and Goto, S. G. (2005). Gene expression of heat-shock proteins (Hsp23, Hsp70 and Hsp90) during and after larval diapause in the blow fly *Lucilia sericata*. *Journal of Insect Physiology* **51**, 641–647.

Tanaka, S. and Zhu, D.-H. (2008). Geographic variation in embryonic diapause, cold-hardiness and life cycles in the migratory locust *Locusta migratoria* (Orthoptera: Acrididae) in China. *Entomological Science* **11**, 327–339.

Tauber, E. and Kyriacou, B. P. (2001). Insect photoperiodism and circadian clocks: models and mechanisms. *Journal of Biological Rhythms* **16**, 381–390.

Tauber, E. and Kyriacou, C. P. (2007). Genomic approaches for studying biological clocks. *Functional Ecology* **22, 1**9-29**.**

Tauber, M. J. and Tauber, C. A. (1972). Geographic variation in critical photoperiod and in diapause intensity of *Chrysopa carnea* (Neuroptera). *Journal of Insect Physiology* **18**, 25–29.

Tauber, M. J. and Tauber, C. A. (1976). Insect seasonality: diapause maintenance, termination, and postdiapause development. *Annual Review of Entomology* **21**, 81–107.

Tauber, M., Tauber, C. and Masaki, S. (1986). *Seasonal Adaptations of Insects*. Oxford University Press, Oxford, UK

Tauber, E., Zordan, M., Sandrelli, F., Pegoraro, M., Osterwalder, N., Breda, C., Daga, A., Selmin, A., Monger, K., Benna, C., et al. (2007). Natural selection favors a newly derived timeless allele in *Drosophila melanogaster*. *Science* **316**, 1895–1898.

Teets, N. M. and Denlinger, D. L. (2013). Physiological mechanisms of seasonal and rapid cold-hardening in insects. *Physiological Entomology* **38**, 105–116.

Terblanche, J. S., Marais, E. and Chown, S. L. (2007a). Stage-related variation in rapid cold hardening as a test of the environmental predictability hypothesis. *Journal of Insect Physiology* **53**, 455–462.

Terblanche, J. S., Deere, J. a, Clusella-Trullas, S., Janion, C. and Chown, S. L. (2007b). Critical thermal limits depend on methodological context. *Proceedings of The Royal Society* **274**, 2935–4292.

Terblanche, J. S., Clusella-Trullas, S., Deere, J. A and Chown, S. L. (2008). Thermal tolerance in a south-east African population of the tsetse fly *Glossina pallidipes* (Diptera, Glossinidae): implications for forecasting climate change impacts. *Journal of Insect Physiology* **54**, 114–127.

Terblanche, J. S., Hoffmann, A. A, Mitchell, K. A, Rako, L., le Roux, P. C. and Chown, S. L. (2011). Ecologically relevant measures of tolerance to potentially lethal temperatures. *Journal of Experimental Biology* **214**, 3713–3725.

Thomas, C. D., Cameron, A., Green, R. E., Bakkenes, M., Beaumont, L. J., Collingham, Y. C., Erasmus, B. F. N., De Siqueira, M. F., Grainger, A., Hannah, L. (2004). Extinction risk from climate change. *Nature* **427**, 145–148.

Thorne, B. L., Breisch, N. L. and Haverty, M. I. (2002). Longevity of kings and queens and first time of production of fertile progeny in dampwood termite (Isoptera; Termopsidae; Zootermopsis) colonies with different reproductive structures. *Journal of Animal Ecology* **71**, 1030–1041.

Touchette, B. and Burkholder, J. (2000). Overview of the physiological ecology of carbon metabolism in seagrasses. *Journal of Experimental Marine Biology and Ecology* **250**, 169–205.

Tougou, D., Musolin, D. L. and Fujisaki, K. (2009). Some like it hot! Rapid climate change promotes changes in distribution ranges of *Nezara viridula* and *Nezara antennata* in Japan. *Entomologia Experimentalis et Applicata* **130**, 249–258.

Tu, M. S. and Dickinson, M. H. (1996). The control of wing kinematics by two steering muscles of the blowfly (*Calliphora vicina*). *Journal of Comparative Physiology A* **6**, 178 - 182.

Turnock, W. I. J. and Fields, G. (2005). Winter climates and cold hardiness in terrestrial insects. *European Journal of Entomology* **102**, 561–576.

Tzanakakis, M. E. (1959). An ecological study of the Indian-meal moth *Plodia interpunctella* (Hubner) with emphasis on diapause. *Hilgardia* **29**, 205–245.

Urquhart, F. and Urquhart, N. (1978). Autumnal migration routes of the eastern population of the monarch butterfly in North America to the overwintering site in the Neovolcanic Plateau of Mexico. *Canadian Journal of Zoology* **56**, 1759–1764.

Vaz Nunes, M. and Saunders, D. S. (1989). The effect of larval temperature and photoperiod on the incidence of larval diapause in the blow fly, *Calliphora vicina*. *Physiological Entomology* **14**, 471–474.

Vesala, L. and Hoikkala, A. (2011). Effects of photoperiodically induced reproductive diapause and cold hardening on the cold tolerance of *Drosophila montana*. *Journal of Insect Physiology* **57**, 46–51.

Vesala, L., Salminen, T. S., Kankare, M. and Hoikkala, A (2012). Photoperiodic regulation of cold tolerance and expression levels of regucalcin gene in *Drosophila montana*. *Journal of Insect Physiology* **58**, 704–709.

Vinogradova, E. B. (1986). Geographical variation and ecological control of diapause in flies. *Proceedings in Life Sciences* **12** . 35–47.

Vinogradova, E. B. (2009). Effect of food and temperature on the reproduction of the blowfly, *Calliphora vicina* R.-D. (Diptera, Calliphoridae), a popular model object in biological research. *Entomological Reviews* **89**, 137–142.

Vinogradova, E. B. (2011). Some principles of selecting natural material for rearing and the endogenous processes in laboratory strains of the blowfly *Calliphora vicina* R.-D. (Diptera, Calliphoridae). *Entomological Reviews* **91**, 1–6.

Vinogradova, E. B. and Zinovjeva, K. B. (1972). Experimental investigation of the seasonal aspect of the relationship between blow flies and their parasites. *Journal of Insect Physiology* **18**, 1629–1638.

Visser, M. E. and Both, C. (2005). Shifts in phenology due to global climate change: the need for a yardstick. *Proceedings of theRoyal Society* **272**, 2561–2569.

Volney, W. J. and Fleming, R. A. (2000). Climate change and impacts of boreal forest insects. *Agriculture, Ecosystems & Environment* **82**, 283–294.

Walther, G.-R. (2010). Community and ecosystem responses to recent climate change. *Philosophical Transactions of the Royal Society of London. Series B, Biological sciences* **365**, 2019–2024.

Walther, G., Post, E., Convey, P., Menzel, A., Parmesan, C., Beebee, T. J. C., Fromentin, J., Hoegh-Guldberg, O., Bairlein, F., Parmesank, C. (2002). Ecological responses to recent climate change. *Nature* **416**, 389–395.

Watanabe, M. and Tanaka, K. (1998). Adult diapause and cold hardiness in *Aulacophora nigripennis* (Coleoptera: Chrysomelidae). *Journal of Insect Physiology* **44**, 1103–1110.

Westgarthsmith, A, Leroy, S., Collins, P. and Harrington, R. (2007). Temporal variations in English populations of a forest insect pest, the green spruce aphid (*Elatobium abietinum*), associated with the North Atlantic Oscillation and global warming. *Quaternary International* **173-174**, 153–160.

Wheeler, C. A. and Cardé, R. T. (2013). Defensive allomones function as aggregation pheromones in diapausing Ladybird Beetles, *Hippodamia convergens*. *Journal of Chemical Ecology* **39**, 723–732.

Williams, C. M., Marshall, K. E., MacMillan, H. A, Dzurisin, J. D. K., Hellmann, J. J. and Sinclair, B. J. (2012). Thermal variability increases the impact of autumnal warming and drives metabolic depression in an overwintering butterfly. *PLoS ONE* **7**, e34470.

Wilson, R. and Franklin, C. (2002). Testing the beneficial acclimation hypothesis. *Trends in Ecology & Evolution* **17**, 66–70.

Wyatt, G. R. and Meyer, W. L. (1959). The chemisry of insect hemolymph III. Glycerol. *The Journal of General Physiology* **42**, 1005–1011.

Wylie, H. G. (1980). Factors affecting facultative diapause of *Microctonus vittatae* (Hymenoptera: Braconidae). *The Canadian Entomologist* **112**, 747–749.

Xu, W.-H., Lu, Y.-X. and Denlinger, D. L. (2012). Cross-talk between the fat body and brain regulates insect developmental arrest. *Proceedings of the National Academy of Sciences of the United States of America* **109**, 14687–14692.

Xue, F., Spieth, H. R., Aiqing, L. and Ai, H. (2002). The role of photoperiod and temperature in determination of summer and winter diapause in the cabbage beetle, *Colaphellus bowringi* (Coleoptera: Chrysomelidae). *Journal of Insect Physiology* **48**, 279–286.

Yi, S.-X. and Lee, R. E. (2011). Rapid cold-hardening blocks cold-induced apoptosis by inhibiting the activation of pro-caspases in the flesh fly *Sarcophaga crassipalpis*. *Apoptosis* **16**, 249–255.

Zachariassen, K. E. (1985). Physiology of cold tolerance in insects. *Physiological Reviews* **65**, 799–832.

Zachariassen, K. E. and Hammel, H. T. (1976). Nucleating agents in the haemolymph of insects tolerant to freezing. *Nature* **262**, 285–287.

Zachariassen, K. E. and Husby, J. A. (1982). Antifreeze effect of thermal hysteresis agents protects highly supercooled insects. *Nature* **298**, 865–867.

Zachariassen, K. E., Kristiansen, E., Pedersen, S. A. and Hammel, H. T. (2004). Ice nucleation in solutions and freeze-avoiding insects-homogeneous or heterogeneous? *Cryobiology* **48**, 309–321.

Zaslavski, V. A. and Umarova, T. (1990). Environmental and endogenous control of diapause in Trichoramma species. *Entomophaga* **35**, 23–29.

Zhang, W., Ricketts, T. H., Kremen, C., Carney, K. and Swinton, S. M. (2007). Ecosystem services and dis-services to agriculture. *Ecological Economics* **64**, 253–260.

Zhang, Q., Lu, Y.-X. and Xu, W.-H. (2012). Integrated proteomic and metabolomic analysis of larval brain associated with diapause induction and preparation in the cotton bollworm, *Helicoverpa armigera*. *Journal of Proteome Research* **11**, 1042–1053.

Zheng, X., Cheng, W., Wang, X. and Lei, C. (2011). Enhancement of supercooling capacity and survival by cold acclimation, rapid cold and heat hardening in *Spodoptera exigua*. *Cryobiology* **63**, 164–169.

Zipkin, E. F., Ries, L., Reeves, R., Regetz, J. and Oberhauser, K. S. (2012). Tracking climate impacts on the migratory monarch butterfly. *Global Change Biology* **18**, 3039–3049.

APPENDICES

Appendix A: Supplementary material for chapter 5

Table A1 The frequency (number of days) that air temperature exceeded, 25°C, 20°C and 15°C and fell below 0°C from the 1st August to 31st October (diapause induction period), 1st November to 31st December (diapause maintenance) and 1st January to 31st March (post diapause quiescence [PDQ] period) between 2009 to 2013 inclusive. Frequencies for September are provided in brackets.

Temperature threshold	Winter period (years)	August to October (diapause induction period	November to December (diapause maintenance period)	January to March (PDQ period)
Temperatures exceeded 25°C	2009 – 2010	0 (0)	0	0
	2010 – 2011	0 (0)	0	0
	2011 – 2012	6 (3)	0	1
	2012 - 2013	0 (0)	0	0
Temperatures exceeded 20°C	2009 - 2010	21 (5)	0	0
	2010 - 2011	16 (4)	0	0
	2011 - 2012	29 (10)	0	1
	2012-2013	21 (10)	0	0
Temperatures exceeded 15°C	2009 - 2010	71 (29)	1	0
	2010 - 2011	59 (23)	1	5
	2011 - 2012	77 (30)	2	9
	2012-2013	52 (30)	0	0
Temperatures below 0°C	2009 - 2010	0 (0)	14	42
	2010 - 2011	2 (0)	30	25
	2011 - 2012	0 (0)	3	21
	2012-2013	2 (0)	14	46

Table A2 The duration (number of hours) that air temperature exceeded, 25°C, 20°C and 15°C and fell below 0°C from the 1st August to 31st October (diapause induction period), 1st November to 31st December (diapause maintenance) and 1st January to 31st March (post diapause quiescence [PDQ] period) between 2009 to 2013 inclusive. Frequencies for September are provided in brackets.

Temperature threshold	Winter period (years)	August to October (diapause induction period	November to December (diapause maintenance period)	January to March (PDQ period)
Temperatures exceeded 25°C	2009 – 2010	0 (0)	0	0
	2010 – 2011	0 (0)	0	0
	2011 – 2012	26 (11)	0	0
	2012 - 2013	0 (0)	0	0
Temperatures exceeded 20°C	2009 - 2010	95 (0)	0	0
	2010 - 2011	55 (0)	0	0
	2011 - 2012	162 (28)	0	3
	2012-2013	116 (54)	0	0
Temperatures exceeded 15°C	2009 - 2010	802 (45)	1	0
	2010 - 2011	625 (41)	6	25
	2011 - 2012	915 (199)	4	56
	2012-2013	637 (327)	0	0
Temperatures below 0°C	2009 - 2010	0 (0)	162	429
	2010 - 2011	11 (0)	552	186
	2011 - 2012	0 (0)	13	270
	2012-2013	9 (0)	118	563

Table A3 The frequency (number of days) that soil temperature exceeded 15°C and 5°C and fell below 0°C from the 1st August to 31st October (diapause induction period), 1st November to 31st December (diapause maintenance) and 1st January to 31st March (post diapause quiescence [PDQ] period) 2009 to 2013 inclusive. Frequencies for September are provided in brackets.

Temperature threshold	Winter period (years)	August to October (diapause induction period	November to December (diapause maintenance period)	January to March (PDQ period)
Temperatures exceeded 15°C	2009 - 2010	76 (30)	0	0
	2010 - 2011	66 (28)	0	0
	2011 - 2012	73 (30)	0	7
	2012-2013	51 (30)	0	0
Temperatures exceeded 5°C	2009 - 2010	92 (30)	44	48
	2010 - 2011	92 (30)	22	64
	2011 - 2012	92 (30)	48	64
	2012-2013	92 (30)	40	36
Temperatures below 0°C	2009 - 2010	0 (0)	2	13
	2010 - 2011	0 (0)	12	0
	2011 - 2012	0 (0)	0	7
	2012-2013	0 (0)	0	0

Table A4 The duration (number of hours) that soil temperature exceeded 15°C and 5°C and fell below 0°C from the 1st August to 31st October (diapause induction period), 1st November to 31st December (diapause maintenance) and 1st January to 31st March (post diapause quiescence [PDQ] period) 2009 to 2013 inclusive. Frequencies for September are provided in brackets.

Temperature threshold	Winter period (years)	August to October (diapause induction period)	November to December (diapause maintenance period)	January to March (PDQ period)
Temperatures exceeded 15°C	2009 - 2010	76 (30)	0	0
	2010 - 2011	66 (28)	0	0
	2011 - 2012	73 (30)	0	7
	2012-2013	51 (30)	0	0
Temperatures exceeded 5°C	2009 - 2010	92 (30)	44	48
	2010 - 2011	92 (30)	22	64
	2011 - 2012	92 (30)	48	64
	2012-2013	92 (30)	40	36
Temperatures below 0°C	2009 - 2010	0 (0)	2	13
	2010 - 2011	0 (0)	12	0
	2011 - 2012	0 (0)	0	7
	2012-2013	0 (0)	0	0

Table A5 The frequency in days (and duration in hours) that temperatures were below the CT_{min} (0.9°C) for adult *C. vicina* in December, January and February 2009 to 2013.

	2009/10	2010/11	2011/12	2012/13
December	17 (242)	**22 (434)**	5 (32)	12 (138)
January	**22 (386)**	17 (209)	10 (125)	17 (298)
February	22 (187)	6 (12)	**13 (216)**	22 (215)

Table A6 The frequency in days (and duration in hours) that temperatures were below the chill coma temperature (-1.7°C) for adult *C. vicina* in December, January and February 2009 to 2013.

	2009/10	**2010/11**	**2011/12**	**2012/13**
December	10 (81)	**20 (312)**	2 (3)	6 (52)
January	**11 (125)**	9 (45)	6 (49)	**10 (79)**
February	8 (3)	0 (0)	**9 (85)**	2 (6)

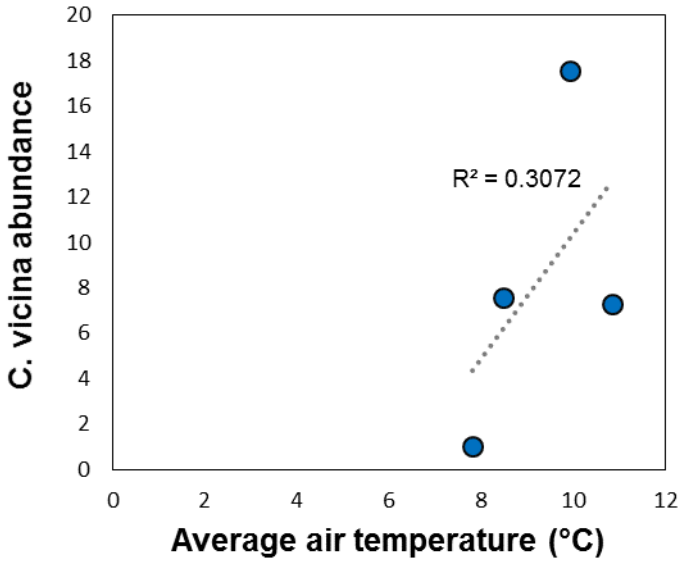


Figure A1 Model 2 regression and R^2 value for the relationship between average *C. vicina* abundance in November and average air temperature in the month directly preceding traps being placed out.

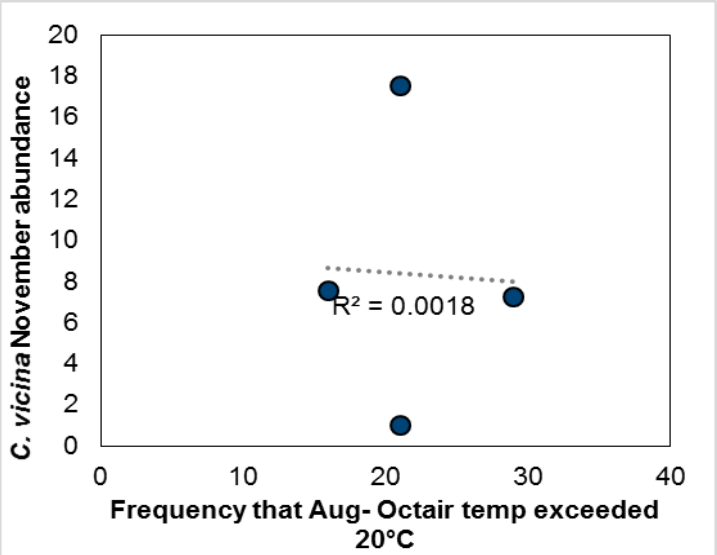


Figure A2 Model 2 regression and R^2 value for the relationship between average *C. vicina* abundance in November and the frequency that air temperature exceeded 20°C between 1st August 31st October, from 2009 – 2012.

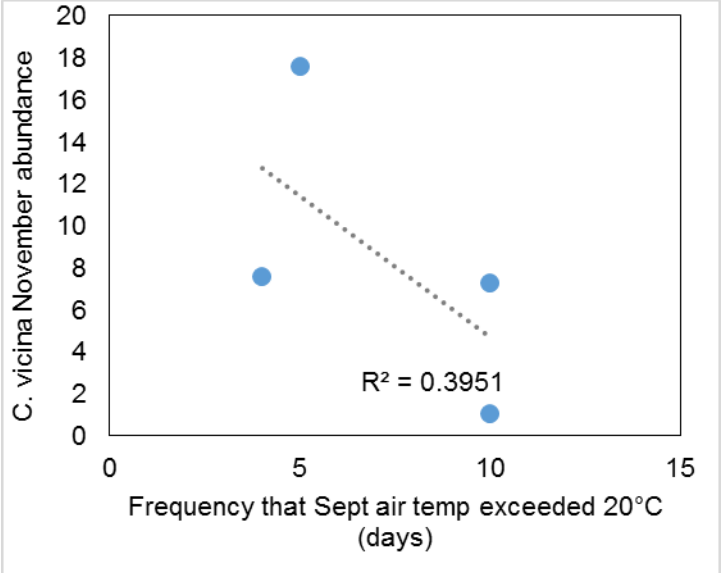


Figure A3 Model 2 regression and R^2 value for the relationship between average *C. vicina* abundance in November and the frequency that air temperature exceeded 20°C in September, from 2009 – 2012.

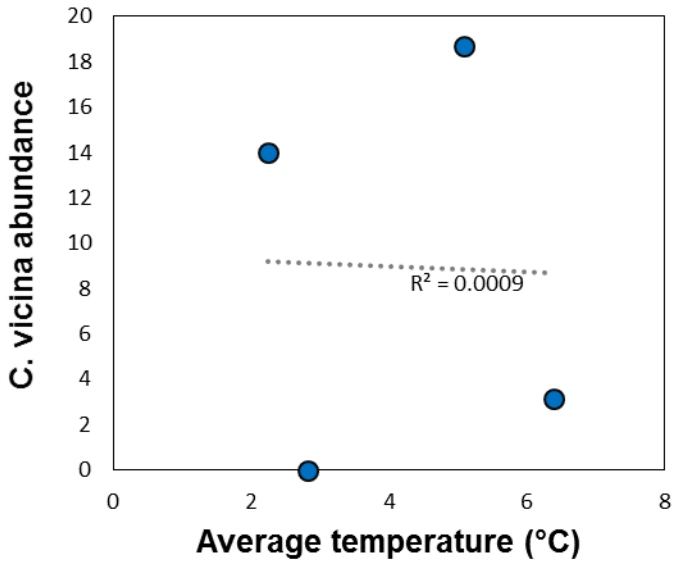


Figure A4 Model 2 regression and R^2 value for the relationship between average *C. vicina* abundance in March and average air temperature in the month directly preceding traps being placed out.

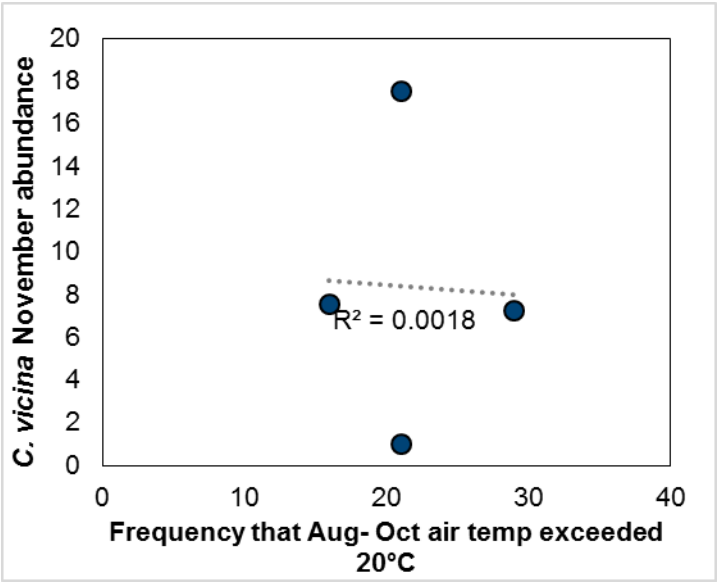


Figure A5 Model 2 regression and R^2 value for the relationship between average *C. vicina* abundance in March and the frequency that air temperature exceeded 20°C between 1st August 31st October, from 2009 – 2012.

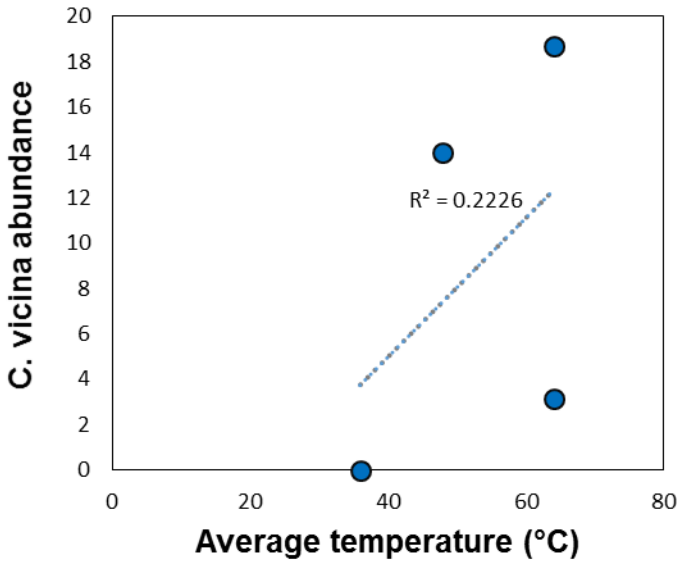


Figure A6 Model 2 regression and R^2 value for the relationship between average *C. vicina* abundance in March and the frequency that soil temperature exceeded 5°C between 1st January and March, from 2009 – 2012.