Rapid cold-hardening and phenology in the hoverfly *Episyrphus balteatus* (Diptera, Syrphidae)

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Abstract

The effect of rapid cold-hardening (RCH) on increasing insect survival at otherwise lethal temperatures is well documented, but its relevance to maintaining insect behaviours at low temperatures remains largely uncharacterised. The hoverfly *Episyrphus balteatus* is weakly cold tolerant but capable of summer migration over long distances to regions in which it may not be able to overwinter in significant numbers, including the UK. RCH was investigated in the laboratory, comparing life stages, sexes, and lethal and behavioural thresholds. RCH significantly increased survival at otherwise lethal sub-zero temperatures in first and second instar larvae, and pupae, but not in eggs, third instar larvae and adults. The capacity for RCH was limited by the relationship between the freezing temperature and that at which chilling injury occurs. RCH did not affect the chill coma temperature in adults (CT_{min} of approximately 0.5°C), but did decrease the low-temperature threshold for flight, by more than 2°C, following hardening at 10°C for periods around 60 minutes or by gradual cooling at 0.5 and 0.1°Cmin^{-1} ($p<0.05$). The flight threshold was significantly lower ($p<0.01$) in females (17.5°C) than in males (19.3°C). The results demonstrate that RCH may have real ecological implications at temperature thresholds relating to behaviour. A record of the phenology of *E. balteatus* at a site in the UK from 1972 to 2001 was also analysed, and significant correlations with temperature identified, including advancing flight period consistent with climate warming.

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Chapter 1

General Introduction

As ectotherms, every aspect of the life history of insects is limited by temperature at some level. Insects in climates that experience seasonal or year-round periods of low temperature must have strategies to overcome these adverse conditions. Such strategies may incorporate elements of (1) behaviour, such as the raising the body temperature by basking or beating the wing muscles, or in the spatial avoidance of low temperatures by migration, (2) physiology, such as the ability to acclimate or enter into diapause, and (3) biochemistry, in the synthesis of cryoprotectant compounds.

1.1 Behavioural avoidance of low temperatures

Many species show behaviours that can increase body temperature in the short term, for example in basking or beating the wing muscles (e.g. Orueta, 2002; Sanborn, 2000). With respect to longer term exposures, such as when overwintering, the nature of the microhabitat of the selected overwintering site can have a significant effect on the temperatures experienced by individuals during the winter (Danks, 1991). Bale (1993) classifies species such as *Musca domestica* as 'opportunistic survivors', overwintering as they do in artificially thermally-buffered microhabitats, such as agricultural buildings or human habitation. In many insects, migration represents a mode whereby low winter temperatures can be avoided entirely. The 'true' migrant[1] species *Danaus plexippus*, the

[1] The literature contains considerable debate surrounding the disambiguation of the terms 'migration' and 'dispersal' in insects. It is now widely the case that 'migration' refers to the behaviour of the individual, and 'dispersal' to the

Monarch butterfly, migrates annually from overwintering sites in Mexico to north-eastern North America, where eggs are laid and the larvae develop during the summer, before the resulting adults of the second generation return south in response to declining temperatures in the autumn. This species has been shown to have only limited cold-hardiness which may constitute an inability to remain in the northern range during the winter (Larsen and Lee, 1994). The extraordinary capacity for long distance migration in this species may thus be seen as a strategy that deals with low temperatures. While other migratory species, such as the many Lepidoptera that show dispersal flight at high altitude, may not demonstrate this degree of control over their destination at the level of the individual, that fact in itself can mean that at the population level, migration enables the location of thermally favourable habitats. The variation in displacements of migrants caused by stochastic variations in wind speed and direction can ensure that migrant populations 'sample' the landscapes over which they fly (Gatehouse, 1994).

1.2 Seasonally-acquired cold hardiness

In insects that cannot avoid low winter temperatures, mechanisms of cold hardiness are required to survive them. Salt (1961) provided a classification of insect cold hardiness strategies: freeze tolerant species survive the formation of ice in fluids and tissues, and freeze intolerant species (now more commonly called 'freeze avoiding') are capable of supercooling to low temperatures without the formation of ice. Species have traditionally

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ecological consequence of that behaviour at the population level (Woiwod *et al*, 2001). However, where the behaviour involved is poorly understood, particularly surrounding the stimuli and responses that initiate and terminate migratory behaviour, it is difficult to distinguish such terminology. This is the case with *E. balteatus* and thus the term 'migration' is used in this thesis to refer to the general movement of this species to and from the UK, without specifically excluding elements of dispersive (or 'vegetative'/ resource-finding) behaviour.

been assigned to either category by cooling individuals to the temperature at which they freeze (supercooling point (SCP)), and determining whether they are killed (freeze avoiding) or can survive at temperatures below this (freeze tolerant).

1.2.1 Freeze tolerance

Freeze tolerant species are able to tolerate the formation of extracellular ice in their body fluids. To assist in this process, ice nucleating agents (INAs) and various cryoprotectants are synthesised. INAs, generally proteins, initiate ice formation in the extracellular fluids. The resultant osmotic gradient causes water to flow out of the cells, leading to a reduced risk of ice forming in the cells. In this way, INAs act to limit supercooling so that nucleation is initiated at a relatively high temperature, resulting in a relatively slow rate of ice formation and small ice crystals, thus reducing the likelihood of injury (Duman, 1982, 2001). INAs are most commonly located in the haemolymph, but have been found elsewhere in some species, such as in the gut of the sawfly *Trichiocampus populi* (Shimada, 1989), and on the extracellular surfaces of organs in the gallfly *Eurosta solidaginis* (Bale *et al.*, 1989).

In addition to INAs, freeze tolerant insects synthesise polyols and sugars which have been shown to prevent mechanical damage, electrolyte imbalances, and other problems caused by the formation of ice (Baust 1973, 1982). Glycerol is the most abundant polyol in insects, and stabilises protein structure, prevents enzyme denaturation, buffers electrolyte concentrations, and prevents secondary recrystallisation during thawing (Zachariassen 1985; Baust 1973, 1982).

Antifreeze proteins (AFPs), also called thermal hysteresis proteins, are also found in freeze tolerant insects. AFPs act to lower the freezing point of a solution relative to its melting point, and thus in insects act to prevent secondary recrystallisation during the warming phase at the end of winter and prior to actual thawing of the frozen tissues and fluids (Duman 2001).

The result of these processes in freeze tolerant species is to give the insect a relatively high sub-zero SCP (typically -5 to -10°C), below which they survive down to a significantly depressed lower lethal temperature (LLT). However, there is, of course, variation within this general pattern of the freeze tolerance mechanism. The most variable aspect of the freeze tolerant group is the degree to which individuals survive below their SCP (Bale, 2002). For example, *Hydromedion sparsutum*, *Syrphus ribesii*, and *Eurosta solidaginis* all freeze above -10°C, but survive to 6, 20 and 40°C below their SCP respectively (Bale *et al.*, 2000; Hart and Bale 1997a; Bale *et al.*, 1989).

Further exceptions to this general pattern of the freeze tolerance mechanism include the formation of intracellular ice observed in nematodes (Wharton and Ferns, 1995) and in fat body cells of the freeze-tolerant larvae of the gallfly *Eurosta solidaginis* (Lee *et al.*, 1993). The beetle *Pytho deplanatus* freezes and survives at very low temperatures (-54°C) because it does not synthesise nucleating agents to initiate freezing at a higher temperature (Ring, 1982). Larvae of the beetle *Osmoderma eremicola* do not accumulate polyols, which may account for their lower lethal temperature being not greatly lower than their freezing temperature (Storey *et al.*, 1993).

1.2.2 Freeze avoidance

Freeze avoiding species are not able to tolerate the formation of ice in their tissues, and so avoid this by supercooling (Salt, 1961). For example, the goldenrod gall moth *Epiblemma scudderiana*, can survive to -38°C without freezing (Rickards *et al.*, 1987). The likelihood of spontaneous ice formation occurring in the supercooled state is reduced by various physiological adjustments, such as the reduction of body water content and increase in body fat content, coupled with the removal or masking of ice nucleating agents, in particular through the cessation of feeding and evacuation of the gut (Neven *et al.* 1986).

Added to this, the accumulation of various cryoprotectants, including polyols and AFPs can further increase supercooling capacity. The colligative effect of polyols can depress the SCP; the high viscosity of glycerol solutions at low temperature may act to inhibit ice nucleation (Salt, 1961). In freeze avoiding insects, AFPs further depress the SCP and stabilise the supercooled state by inhibiting ice nucleation and inoculative freezing (Zachariassen and Hammel, 1976; Duman, 1982; Duman and Horwath, 1983).

1.2.3 Environmental cues

Four main environmental cues stimulate the accumulation of cryoprotectants in insects, to a lesser or greater degree in individual species: temperature, photoperiod, hydration level, and nutrient balance (Baust, 1981, 1982). In freeze tolerant insects, ice nucleating agents are normally synthesised in early winter, in response to decreasing temperature, and lost in spring (Zachariassen, 1982, 1985; Baust, 1982). In both freeze tolerant and freeze

avoiding species, levels of polyols and sugars increase in the autumn and decline in the spring (Zachariassen, 1979, 1980). AFPs are typically present from early autumn to spring, and, unlike glycerol, are not lost in short periods of warm weather (Duman *et al.*, 1982). AFPs may therefore protect against the effects of freezing in early autumn and recrystallisation during thawing in spring.

However, not all changes in cold hardiness are environmentally induced. For example, Worland *et al.* (2006) found significant variation in SCP between individuals of the collembolan *Ceratophysella denticulata* on different days, and at different sites on the same day. This variation was attributed to the decrease in SCP during moulting, when the mid-gut lining is shed with the consequent evacuation of ice-nucleating gut contents; the SCP was again elevated on the commencement of feeding after completion of the moult. Moulting also decreased SCP in the collembolan *Tullbergia antarctica* (Worland, 2005). These species moult regularly as part of the growth process through the summer season, and individuals thus experience periods of increased cold hardiness independent of any particular environmental cue (Worland *et al.*, 2006).

1.2.4 Further classifications

The freeze tolerant/avoiding classification is suitable for discussing insect responses to freezing. However, Bale (1987, 1993) showed that for the freezing temperature to form a meaningful division between tolerance and avoidance strategies, the species in question must remain alive down to this temperature, but that this is not the case with the majority of overwintering insects, which die at temperatures above this (pre-freeze mortality). Bale (1993) thus introduced the concepts of chill tolerance and chill susceptibility, to describe

the responses of species to chilling injury and pre-freeze mortality. For example, many aphids have very low supercooling points, but die after only very short periods of time at higher sub-zero temperatures, as seen in *Myzus persicae,* where the supercooling point is -25°C, and yet no individuals survived 1 minute exposures at temperatures between -5 and -15°C (Bale *et al.*, 1988). This species is thus highly chill susceptible. By contrast, in the chill tolerant mite *Alaskozetes antarcticus*, where the supercooling point is -30°C, 73% of individuals survived 100 days at -20°C (Cannon, 1987).

A link between desiccation resistance and cold tolerance has also been recognised. For example, an increase in cold hardiness associated with water loss has been described in *Onychiurus arcticus* (Worland *et al,*. 1998; Holmstrup and Somme, 1998), and *Pringleophaga marioni* (Sinclair and Chown, 2003). This led Sinclair *et al* (2003) to describe a "third strategy" of cold hardiness (relative to the 'two' of freeze tolerance and avoidance), termed cryoprotective dehydration, whereby water is lost from the tissues to ice in the surrounding environment as temperature decreases, thereby maintaining the animal in vapour pressure equilibrium with its environment, preventing freezing down to -30°C, in spite of a summer SCP of -6.5°C in *O. arcticus*. Indeed, it has been suggested that supercooling ability may, at least in part, be the result of adaptation to desiccation resistance, rather than low temperature *per se* (Renault *et al*, 2002).

1.2.5 Changes of strategy

Some species show changes in their cold hardiness strategy over successive low temperature cycles. Horwath and Duman (1984) reported that wild-collected larvae of the beetle *Dendroides canadensis* were freeze tolerant during two successive winters, but

freeze avoiding the next. Lower lethal temperatures (LLTs) were similar in all years, but in the 'freeze-avoiding year' supercooling points were lower, at a level equivalent to the LLT (by as much as 18°C, from around -10 to -26°C). This is consistent with a classically freeze-avoiding strategy. Causal factors were unclear, although a concurrent loss of ice nucleating proteins was observed. The same study subsequently found the same phenomenon in larvae of another beetle, *Cucujus clavipes.*

A dual strategy has also been observed in the sub-Antarctic beetle *Hydromedion sparsutum* (Bale *et al.*, 2001). Here it is suggested that, following exposure to freezing temperatures early in winter, the population may become segregated into two sub-populations, the first remaining weakly freeze-tolerant and able to survive multiple freeze-thaw cycles, and the second, with SCPs which are either lower initially or which are depressed, extending supercooling but becoming more likely to be killed by freezing. In the latter group, substantial depression of the SCP apparently only occurred when larvae were exposed to sub-zero temperatures but did not actually freeze.

Recently, Brown *et al.* (2004) found that in larvae of the hoverfly *Syrphus ribesii* stored at 2°C for up to 150 days, some individuals lost freeze-tolerance, supercooled to lower temperatures than normal (down to -22°C compared to around -10°C), and were killed by freezing. A similar observation was made when larvae were exposed to multiple freeze-thaw cycles (freezing at daily or weekly intervals), when some larvae were found to have lost freeze-tolerance after the first freezing event, and apparently adopted a freeze-avoiding strategy during subsequent freezes. Here, then, a single freezing event, presumably the first that the larva experiences, might be the trigger stimulating the change in strategy.

1.3 Rapid cold-hardening

1.3.1 The rapid cold-hardening process

The above processes mostly relate to long-term or seasonal acclimation associated with overwintering. In contrast, rapid cold-hardening (RCH) is a process whereby cold tolerance is significantly increased after only brief periods of acclimation (minutes or hours). The term 'rapid cold-hardening' was first used by Lee *et al* (1987) to describe this process in larvae and pharate adults of the flesh fly *Sarcophaga crassipalpis* (and three other species), although Mellanby (1939) identified a similar process after slightly longer hardening durations (20 hours) in the cockroach *Blatta orientalis* ("acclimatization is not a long process"), as did Meats (1973) in relation to the torpor temperature of the fruit fly *Dacus trioni* ("rapid acclimatization"). Lee *et al* (1987) found that, in non-diapausing *Sarcophaga crassipalpis*, few individuals (<5%) survived an exposure of 2h at -10°C. However, chilling for 30min at 0°C before exposure doubled the survival rate, and 2h at 0°C before exposure increased survival to more than 88%.

Lee *et al* (1987) also examined RCH in *S. crassipalpis* reared under diapause-inducing conditions, and these pupae also showed the response: no survival after 1d at -17°C, but 90% survival when this was preceded by 2h at 0°C. Diapausing individuals gradually accumulate cryoprotectants during the first month after pupariation, and it was thus concluded that RCH may confer protection early in diapause for overwintering insects, before full seasonally-acquired protection is in place.

Further studies characterised other aspects of the RCH process. The 'additional' cold hardiness conferred by RCH is generally lost as rapidly as it was gained, on returning the individual to the original rearing temperature (e.g. *Musca domestica* (Coulson and Bale, 1990), and pine needle gall midge *Thecodiplosis japonensis* (Li *et al.*, 1999)). Increasing the duration of acclimation increases subsequent survival at the sub-zero exposure, but only up to a point, beyond which the acclimation period itself may increase mortality, even without subsequent sub-zero exposures; in other words there is an optimum acclimation time (Coulson and Bale, 1990).

Most species in which RCH is described are chill-susceptible; mortality at low temperatures occurs well above the supercooling point. RCH thus provides protection against cold shock injury, which occurs at temperatures above the supercooling point (Morris *et al.*, 1983), thereby increasing cold tolerance in chill-susceptible insects. It was furthermore suggested that RCH might allow non-overwintering individuals to respond to diurnal temperature changes, given the short times over which it develops and is lost.

A response to cold shock is of clear benefit to chill susceptible insects, but RCH has also been shown in classically freeze-avoiding polar species, which do not succumb to chill injury at relatively high sub-zero temperatures but show high levels of survival down to the supercooling point. A process of this type was identified in three species of Antarctic microarthropod (*Alaskozetes antarcticus, Cryptopygus antarcticus* and *Halozetes belgicae*) by Worland and Convey (2001). Here, the supercooling point both decreased and increased over periods of hours, in response to changes in ambient temperature, in laboratory and field conditions. The greatest responses occurred at temperatures appropriate to the diurnal temperature range of these species' microhabitat, further

suggesting a role for RCH in response to daily temperature changes. Worland and Convey (2001) also hypothesise that this process might represent a saving in metabolic costs, in preventing the individual from entering prematurely into more expensive, seasonal cold hardiness (in diapause or otherwise) in response to a false cue.

RCH has also been tested for in larvae of the freeze *tolerant* sub-Antarctic caterpillar *Pringleophaga marioni* (Sinclair and Chown, 2003). Here, the classic response did not occur (no acclimation at low temperatures increased the survival rate after freezing). The authors conclude that year-round freeze tolerance may fulfil the same requirements as RCH does in other species. However, Lee *et al.* (2006) found that RCH did increase survival while frozen in larvae of the freeze tolerant midge *Belgica antarctica*. RCH in freeze tolerant species is a new field of enquiry.

1.3.2 Rates of cooling

An obvious criticism of an experimental protocol involving direct transfer from summer conditions to subzero temperatures is that such rates of cooling are not prevalent in nature. A number of studies have addressed this issue. Kelty and Lee (1999) rapidly cold-hardened adults of *D. melanogaster* by cooling at either "natural" rates of 0.05 and 0.1°C/min, or faster rates of 0.5 and 1°C/min. Survival of a subsequent cold shock was significantly greater in flies cooled at natural rates, suggesting that RCH may function more strongly in nature than is shown in direct transfer experiments. Gradual cooling has been found to induce RCH in most species investigated, including for example the flower thrips *Frankliniella occidentalis* at rates between 0.1 and 0.5°C/min (McDonald *et al.*, 1997), and in the fruit fly *Bactrocera oleae* at 0.4°C/min (Koveos, 2001). In *Musca*

domestica however, RCH was induced by gradual cooling from 20°C to 0 or –7°C at rates up to 0.1°C/min but no higher, implying that RCH is a function of time spent at an acclimating temperature (or within an acclimating range), rather than the cooling process itself (Coulson and Bale 1990). This conclusion was supported by work with *F. occidentalis* which showed no difference in effect between (a) cooling (at up to 1°C/min) from 20°C all the way down to the exposure at –11.5°C or (b) cooling to 0°C and transferring directly from there to –11.5°C, suggesting a lower limit for inducing RCH of 0°C (McDonald *et al.*, 1997). This suggests that experiments involving direct transfer do often, in fact, elicit the same effects as cooling at more natural rates, provided the time spent within the acclimating range is the same.

1.3.3 Mechanisms: glycerol, heat shock proteins and membrane lipids

Glycerol is known to be an important cryoprotectant synthesised by both freeze tolerant and avoiding species, acting osmotically to lower the freezing temperature of body fluids (Baust 1973, 1982). Glycerol also stabilises protein structure and prevents enzyme denaturation, and buffers electrolyte concentrations (Zachariassen 1985; Baust 1973, 1982). Lee *et al* (1987) recorded a 2 to 3-fold increase in glycerol levels in larvae and pupae of the flesh fly *Sarcophaga crassipalpis* after rapid cold-hardening at 0°C for 2h. Although it was acknowledged that the magnitude of this increase would be insufficient to fully account for the increase in cold hardiness by colligative effects alone, many subsequent studies suggest glycerol accumulation as a possible mechanism underlying RCH.

Anoxia may also induce RCH. For example, in *M. domestica*, 1-3h in an atmosphere of oxygen-free nitrogen significantly increased survival at –7°C, although acclimation at 0°C was more effective as a hardening treatment (Coulson and Bale, 1991). These results suggest that simple metabolic suppression may have a role in RCH. Coulson and Bale (1991) correlated reduced ATP levels in anoxic *M. domestica* with the RCH induced by anoxia. Other energy substrates are also known to be important to cold tolerance in general, and are reduced in cold-shocked individuals (Misener *et al.*, 2001).

A number of studies have highlighted similarities between the responses to cold shock and to heat shock in insects, and suggested the involvement in RCH of proteins previously associated with the response to heat shock in insects: so-called 'heat shock proteins' (HSPs). For example, when *S. crassipalpis* are acclimated at 40°C prior to exposure at 45°C, there is an increase in survival over that following direct transfer, a heat-hardening process which is superficially very similar to RCH (Chen *et al.*, 1990). High temperature acclimation also protected against cold shock (i.e. rapid acclimation at 40°C results in increased survival after exposure to –10°C). This 'cross-protection' occurs in other species, for example *D. melanogaster* (Sejerkilde *et al.*, 2003). Joplin *et al* (1990) found in *S. crassipalpis* that brief exposures to –10 or –18°C induced the production of the HSP compliment normally associated with protecting against heat shock in this species. This suggested that HSPs may be part of a generalised response protecting against physiological damage from extremes of temperature, both high and low.

However, Joplin *et al* (1990) also found that HSP expression continues in *S. crassipalpis* for up to 6h after cold shock, on return to 25°C, a fact which is inconsistent with the loss of RCH within 2h of return from the sub-zero exposure. Misener *et al.* (2001) showed that

when *D. melanogaster* were treated with a protein synthesis inhibitor, the RCH response remained unaffected, even though the survival rate following direct transfer without acclimation was reduced. This evidence suggests that protein synthesis is less important for RCH than for intrinsic cold shock tolerance. The results of some other studies on HSPs are conflicting. For example, Kelty and Lee (2001) found that expression of Hsp70 (and, indeed, glycerol level) was not increased in rapidly cold-hardened *D. melanogaster*, while Sejerkilde *et al.* (2003) did record increased Hsp70 expression in this species after RCH.

The fact that RCH can be induced by anoxia and acclimation at high temperatures, as well as at low temperatures, may suggest that RCH may only be one aspect of a wider, general physiological response to metabolic stress, involving HSPs. However, across insect taxa, it is widely observed that species' show considerably less geographical variation in upper than in lower thermal thresholds, and many species do not show acclimation at the upper threshold to the same degree as widely observed at the lower threshold (Chown, 2000). The induction and operation of HSPs during cold shock are poorly understood, and, in the absence of a confirmed common mechanism, the similarities between heat shock and cold shock responses may be only superficial (Sinclair and Roberts, 2005).

Most recently, changes in the lipid composition of cell membranes during RCH have been observed in *Drosophila melanogaster* (Overgaard *et al.*, 2005), and linked to increases in membrane fluidity in fat body cells of rapidly cold-hardened *Sarcophaga crassipalpis* (Lee *et al.*, 2006). Levels of oleic acid in particular were found to increase in *S. crassipalpis* cell membranes during both RCH and entry into diapause (Michaud and Denlinger, 2006). Such changes prevent cell membranes from becoming increasingly

rigid as temperature decreases, instead maintaining their liquid crystalline state and allowing normal membrane processes to continue, a process known as homeoviscous adaptation (Sinenksy, 1974). Newly identified in the context of RCH, this process is likely to be a key underlying mechanism.

1.3.4 Ecological significance

Clearly, RCH protects against chilling injury at sub-zero temperatures above the supercooling point, thus increasing cold tolerance in chill-susceptible insects. The rapidity of the process established two ideas of an ecological role for RCH early on: (1) allowing insects to track diurnal temperature change (regardless of seasonal cold hardiness), and (2) conferring additional cold tolerance to overwintering individuals at the beginning and end of the season (autumn and spring) when the mechanisms of seasonal cold hardiness are not fully developed or are waning.

Given that RCH appears to provide a response to daily thermoperiod cycles and unpredictable or short-term sub-zero exposures, it seems intuitively sensible that this may also apply to temperature fluctuations experienced in a geographical plane. Migratory insects face an additional set of challenges with regard to low temperature – there may be significant differences between the origin and destination climates, and the route between the two may pass through variable and unpredictable thermal environments. However, few studies have examined cold tolerance in the context of insect migration. The Monarch butterfly *Danaus plexippus* is freeze-susceptible, and is able to rapidly cold-harden, in addition to acquiring seasonal cold tolerance before its autumn migration from eastern

North America to Mexico (Larsen and Lee, 1994). However, evidence of a role for RCH in the migratory process in insects remains limited.

The interconnections between RCH, the heat shock response, and general metabolic suppression raise questions as to whether RCH itself is an adapted mechanism *per se* or part of a generalised stress response. However, adaptation in the capacity to survive cold shock is known to occur. When *Drosophila melanogaster* were selected for cold resistance by undergoing RCH followed by sub-zero exposure (2h at 4°C followed by 80-120min at -7°C), selection for 10 generations resulted in an 82% increase in the capacity of the flies to rapidly cold-harden (% increase in cold shock tolerance after RCH over cold shock tolerance involving direct transfer) (Chen and Walker, 1993). This supports the idea that RCH is in fact an adaptive phenomenon, and that environmental temperature regimes have a role in the evolution of insect cold hardiness mechanisms. This is further reflected in differences in the RCH ability of species from different climatic regions. Chen *et al.* (1990) tested the ability of five species of flesh fly to undergo RCH; two temperate species and one high-altitude tropical species showed a typical RCH response, but two tropical lowland species did not. Temperature extremes are greater in temperate and tropical upland zones than in tropical lowland environments, and this is likely to be reflected in the species' adaptations. With regard to the biochemistry of RCH, it is interesting to note that no glycerol was recorded in the two tropical lowland species, and that all five species showed a classic heat shock response.

Studies of the knock-on effects of undergoing RCH shed further light on the adaptive significance the phenomenon. Most studies of insect cold hardiness assess survival by scoring normal behaviour or response to stimuli, usually not very long after treatment,

while the effects of cold shock can occur some time later (Baust and Rojas, 1985). In contrast, effects of treatment on the reproductive success of survivors, or even on subsequent generations, may have more ecological relevance. Coulson and Bale (1992) examined this in *M. domestica*. RCH conferred much higher survival after cold shock, and thus greater reproduction at the population level, compared to unacclimated individuals. However, female longevity and egg viability were reduced in individuals that were acclimated at 0°C but not cold-shocked (i.e. rapidly cold-hardened only) compared to controls maintained at the rearing temperature.

This implies a trade-off between a metabolic cost of undergoing RCH and the protection it confers. However, recent studies have suggested the converse to be the case in the aphid *Sitobion avenae*. Here, longevity and fecundity were the same in summer-acclimated aphids that had undergone RCH treatment at 0°C (without subsequent sub-zero exposure) as in control groups at 20°C. In other words, undergoing RCH *per se*, had no detrimental effects on longevity or fecundity (Powell and Bale, 2004). Furthermore, in cold-acclimated aphids, individuals that had undergone RCH treatment and also subsequent subzero exposure showed longevity and fecundity equal to that of aphids at 20°C, and even increased levels in some cases (Powell and Bale, 2005).

Both *M. domestica* and *S. avenae* are non-diapausing species. *M. domestica* is not strongly cold tolerant, avoiding low temperatures by opportunistic survival in thermally buffered overwintering sites (Bale, 1993). However, anholocyclic clones of *S. avenae* continue reproduction through the winter, so if RCH is a key aspect of this species' cold hardiness, it might not be expected to impair reproduction, and Powell and Bale (2004, 2005) conclude that there are "no ecological costs associated with rapid cold-hardening in

terms of development, longevity or fecundity" in this species. This identifies a third area in which RCH may have a key ecological role, namely preserving reproductive function in species which do not pass the winter in diapause or quiescence (such as aphids which have short generation times).

1.3.5 Rapid cold-hardening and other thermal thresholds

Most studies of RCH have measured its effects in preventing injury during cold shock. Much less is known about RCH at thresholds other than the lethal limit. Meats (1972) described a rapid acclimation process with respect to torpor temperature in the fruit fly *Dacus tryoni*. No acclimatisation of torpor temperature took place on direct transfer from the rearing temperature, but at cooling rates up to 1°C/min, acclimatisation was effectively immediate and torpor temperature decreased in relation to ambient temperature. RCH also reduces torpor temperature in *Sitobion avenae* (Powell and Bale, in press), and in *Drosophila melanogaster* when cooled at natural rates and in cycling natural thermoperiods (Kelty and Lee, 1999, 2001). It allows cold-shocked *S. crassipalpis* to continue normal grooming behaviour and maintain motor neurone conductivity, while these were severely impaired in individuals that had not undergone RCH (Kelty *et al.*, 1996).

These findings suggest a role for RCH beyond that of preventing mortality from chilling injury. There is a growing line of reasoning that RCH has a broad role in fine-tuning the organism's physiological state in response to normal changes in ambient temperature (Kelty and Lee, 2001; Shreve *et al.*, 2004).

Whether RCH is a specifically-adapted response to cold shock injury or an aspect of a generalised stress response, it clearly has an important role in generating plasticity of cold tolerance in insects, and has been detected in most species so far investigated. However, the majority of studies to date have focused on (1) individual stages of the life cycle, particularly when there is an identifiable overwintering morph, and (2) RCH's effects on reducing chill injury mortality. Opinions about the ecological significance of the phenomenon are diverse, and there are many areas, such as at non-lethal, behavioural thresholds, where the importance of RCH as remains to be fully investigated.

1.4 Applications of research into low temperature biology

The study of low temperature biology in insects has found application in a number of areas, particularly relating to insect pest management in agriculture, but also in the prediction of global warming effects and the potential genetic modification of crop plants.

Temperature is one of the key determinants of insect phenology, and the modelling of phenological and climate data can result in useful predictive models of, for example, pest aphid migration (Zhou *et al.*, 1995; Worner *et al.*, 1995; Werker *et al.*, 1998; McVean *et al.*, 1999). Such research can enable annual predictions of aphid flight dates to be made available to producers. For example, the Scottish Agricultural Science Agency uses suction trap data to monitor the occurrence of *Myzus persicae* and *Macrosiphum euphorbiae* (aphids vectors of viral diseases in seed potato crops) and predict their spring flight dates based on temperatures in the preceding January and February; these predictions are then made available online (Scottish Agricultural Science Agency, 2006).

The introduction of non-native insect predators for use as biocontrol agents in glasshouses can represent a threat to native species, should the alien species establish in the new location (Bale and Walters, 2001). Most such introductions to the UK are of Mediterranean or tropical origin, and, historically, there has been an assumption that they will lack the necessary cold tolerance to survive outside the glasshouse environment through the UK winter. However, this assumption has been shown to be incorrect in a number of cases, such as that of the mirid bug *Macrolophus caliginosis* (Hart *et al.*, 2002). Laboratory measures of cold hardiness, including developmental thresholds and rates at given temperatures, lower lethal temperatures and times, and the ability to acclimate to low temperatures, can enable the assessment of overwintering potential of non-native species before their introduction, and allow recommendations to be made on the licensing of their use (Bale and Walters, 2001; Hatherly *et al.*, 2005). This research is closely linked to current discussions within the European Union on increasing the use of biocontrol without endangering native ecosystems.

Other applications of cold hardiness research to pest management include: the identification of bee species of potential use as pollinators (Corbet *et al.*, 1993); the use of ice-nucleating bacteria and fungi for the control of insect pests (Lee *et al.* 1992, 1994, 1998; Fields, 1993; Fields *et al.*, 1995); the development of cold storage protocols in the mass production of biocontrol agents (Morrison and King, 1977; Caesartonthat *et al.*, 1995; Leopold, 1998; Tezze and Botto, 2004; Lopez and Botto, 2005); and the design of temperature regimes in livestock housing or product storage units to limit insect activity (Hansen and Hara, 1994; Lysyk, 1995; Chervin *et al.*, 1997).

The great constraint put on insect life histories by low temperature has become of particular interest in understanding the effects of global climate change. For example, northward shifts in species' ranges have been described in a growing number of insects (Parmesan *et al.*, 1999), as have changes in phenology (Penuelas and Filella, 2001; Sparks and Menzel, 2002), associated with climate warming. Better understanding of the role of temperature in limiting life history parameters is necessary to improve prediction of future changes (Bale *et al.*, 2002).

Understanding of the genetic basis of insect cold hardiness remains relatively limited, but potential future applications include the genetic modification of crop plants using insect genetic material (Duman, 2001). To this end, antifreeze proteins have been expressed in a number of species, with greatest success using DAFP-1 from the beetle *Dendroides canadensis*. This AFP significantly increased cold hardiness in transgenic *Arabidopsis thaliana* (Huang *et al.*, 2002), as well as in *Drosophila melanogaster* (Nicodemus *et al.*, 2006).

1.5 Hoverflies

Hoverflies are dipterans of the family Syrphidae, and demonstrate a very wide range of habitats, feeding habits and life histories. Around 250 species have been recorded in the UK (Gilbert, 1993). Adults feed primarily at flowers, on nectar and pollen, and the group as a whole are considered important pollinators. Many species, belonging to the sub-family Syrphinae, have aphidophagous larvae, and the bulk of research to date relates to their importance as natural enemies of aphid pests (Chambers and Adams, 1986). This includes methods by which agricultural regimes can be adapted to benefit hoverfly

populations (Cowgill *et al.*, 1993) and the hoverflies' potential for use as biological control agents (Chambers, 1986).

In addition, a number of hoverfly species are considered of conservation importance in the UK, such as the Dartmoor bog hoverfly *Eristalis cyptarum*, and the aspen hoverfly *Hammerschmidtia ferruginea* in Scotland, both subjects of national species action plans.

1.5.1 *Episyrphus balteatus* (DeGeer)

E. balteatus is an abundant hoverfly throughout its range, which covers western Europe, extending from the Mediterranean in the south to northern Scandinavia. The larvae are generalist aphidophagous predators, feeding on a wide range of aphid species. In nature, the hoverfly is known to associate with colonies of the sycamore aphid *Drepanosiphum platanoides*, but in agricultural landscapes is often abundant in crops, particularly cereals, and is of economic importance as a key natural predator of a number of pest aphids (Chambers and Adams, 1986). *E. balteatus* has also been supplied commercially as a glasshouse biocontrol agent (Koppert UK, 2004).

Patterns of migration and overwintering of *E. balteatus* across its range are not well understood. It is present year-round in the southern parts of its range, and extensive movements northward in spring, and southward in autumn, have been recorded in certain areas (Aubert *et al.*, 1976; Aubert and Goeldlin, 1981; Gatter and Schmidt, 1990). Gatter and Schmidt (1990) trapped *E. balteatus* during northward (spring) and southward (autumn) movements in SW Germany, and Aubert and Goeldlin (1981) used mark-recapture techniques to follow migrants for up to 160km through the Alps. In the UK,

adults are present in numbers from April to November approximately, but this varies year to year (Gilbert, 1993; Ball and Morris, 2000). In years of high abundance, swarms are often reported along the south coast of England in the spring, with large numbers of individuals settling on beaches and being washed ashore (Gilbert, pers. comm.). During large southerly movements of insects at Spurn Point in Yorkshire, *E. balteatus* was the most abundant hoverfly caught (Owen, 1956; Sutton, 1969). Hondelmann *et al.* (2005) provided further evidence for substantial long-distance migration by showing extensive gene-flow and genetic similarity between populations from around Europe.

The species is multivoltine, with between 1 and 3 generations possible in the UK based on laboratory measures of its development rate (Hart *et al.*, 1997), but the number of generations has not been observed directly in nature. In certain areas (e.g. evidence from Switzerland), some individuals may overwinter without migrating, and where this occurs the overwintering morph is thought to be the mated female, which may or may not be in reproductive diapause (Schneider, 1969). It has also been suggested that some adults and/or larvae may overwinter in the UK as opportunistic survivors in, for example, agricultural buildings, but it is not known if this represents a significant proportion of the population (Stubbs, pers. comm.), and *E. balteatus* is not thought to overwinter in the UK in significant numbers.

Hart and Bale (1997b) profiled the survival of adult *E. balteatus* at low temperatures, and identified a general lack of inherent cold hardiness or ability to acclimate. For example, survival was only 10% after 10 days at temperatures from 5 to -5°C, and was zero after 10 weeks in UK winter field conditions. This suggests that *E. balteatus* lacks sufficient cold hardiness to overwinter in the UK. Certainly, *E. balteatus* appears later in the summer

than overwintering species, and is widely described as migratory in this country (Ball and Morris, 2000). However, evidence for its movements, and the overwintering ability of life stages other than the adult, remains limited.

1.6 Project Aims

1. Compare the cold hardiness of different life cycle stages

Investigate RCH in all life stages *E. balteatus*, and examine the limits placed on RCH capacity by other determinants of cold hardiness, particularly the SCP (Chapter 3).

2. Differentiate between the sexes in adults

Compare males and females with respect to RCH ability (Chapters 3-5) and phenology (Chapter 6).

3. Investigate the effect of RCH on behavioural thresholds

Determine low-temperature thresholds for flight (Chapter 4) and chill coma (Chapter 5) in *E. balteatus*, and measure any effect of RCH on these thresholds.

4. Relate this information, with climate data, to phenology

Examine the role of temperature in determining annual phenology and abundance of *E. balteatus* at a site in the UK, and the degree to which recent climate warming has altered these (Chapter 6).

This project aims to respond to increasing recognition (e.g. Chown and Storey, 2006) of the current need for better connectivity in the study of insect thermal biology, between

basic physiological process and their ecological effects. The process of RCH will be characterised first at the lethal threshold, then at behavioural thresholds. The consequences of variation in hardening capacity between life stages, sexes, and lethal versus behavioural thresholds will then be linked to the ecology and phenology of the species.

Chapter 2

General Methods

2.1 Introduction

This chapter details the techniques used to rear *Episyrphus balteatus* in the laboratory, and provides a summary of the methods used to quantify cold hardiness parameters, including the general experimental design of rapid cold-hardening experiments.

2.2 Culture of *Episyrphus balteatus*

2.2.1 Collection

To establish the laboratory culture, gravid females were collected while feeding at flowers in the botanic gardens of the University of Birmingham, UK. Further individuals were collected and added to the culture during the summers of the three years for which the project ran. The abundance of individuals in the wild varied considerably from season to season. In 2005, only 30 were collected, while 80 to 100 were collected in other years. To further supplement the culture, out of season or when few wild flies were available, collections of eggs were received from a laboratory culture of UK origin, maintained at Central Science Labs, York, UK.

2.2.2 Adult hoverflies

This project followed the method of Hart and Bale (1997c) (see also Frazer, 1972), with a number of modifications, including a higher ambient temperature and increased variety of aphid prey species. The hoverfly and aphid cultures were maintained at 22°C, 18L:6D photoperiod, and 60-70% RH.

Adults were kept in large (60 x 60 x 95cm high), muslin-sided cages. In nature, the species feeds mainly on pollen and nectar from flowers, and to a lesser extent on the honeydew of the aphids in whose colonies female *E. balteatus* lay their eggs. In the laboratory, food was provided as ground bee pollen (Apitherapy Products Ltd., UK) and sugar cubes in petri dishes, on a feeding stand. Initially, a 50% solution of honey (organic) was provided on cotton wool, as this has been reported as a preferable food source for other Syrphids in captivity, but sugar cubes were subsequently found to be adequate in this case. The food was renewed every few days as necessary.

Water was provided on cotton wool in a petri dish supported on a conical flask; a cotton wool wick drew water from the flask up into the dish. This water fount could be cleaned and filled fortnightly, preventing the need for daily renewal of the water supply. Misting of the muslin cages with water was also carried out every few days; the flies were observed to readily drink from the droplets on the muslin.

2.2.3 Aphid culture

At weekly intervals, 44 seed trays (20x15cm) were thickly sown with seeds of the field

bean *Vicia faba* var. Clipper (King's Seeds, Essex, UK). When the bean seedlings reached approx. 5cm high, the trays were inoculated with aphids and grown on in 150x60x40cm, muslin-sided cages.

Three species of aphid were maintained in culture: the black bean aphid *Aphis fabae*, the vetch aphid *Megoura viciae* and the pea aphid *Acyrthosiphum pisum*. The latter two species are larger than *A. fabae*, and hoverfly larvae were found to develop slightly more quickly when reared on them. However, *A. fabae* was also found to grow significantly faster in culture, and numbers of this aphid could be built up quickly, enabling a faster turnover of bean plants and more aphids available for rearing hoverfly larvae when necessary. Having more than one species of aphid in culture also insulated against occasional loss of aphid cultures due to parasitoid infection. All three species were therefore used for rearing hoverfly larvae for maintenance of the laboratory culture. However, variations in diet can affect cold hardiness in some species, where differences in ice nucleator content (especially bacteria) in the food, and consequently in the individual's gut, may be responsible for differences in supercooling point (e.g. potworm cocoons (Bauer *et al.*, 2001)). For this reason, hoverflies for use in experiments were reared on *A. fabae* only.

2.2.4 Rearing hoverfly larvae

For the collection of eggs, individual seed trays of aphid-infested bean seedlings were placed in the adult cage. Females were observed to lay more readily on bean seedlings that had fewer aphids but plenty of honeydew on the leaves. Depending on the number of adults present, a period of up to 24h was appropriate to collect sufficient eggs on a single

seedling tray to rear on for experiments or culture maintenance.

Larvae were reared in small cages, accommodating 4 or 8 seed trays. As the aphids became depleted, new trays were added and/or the larvae transferred manually to new trays, until pupation. At this point, the seed trays were no longer watered, to limit growth of mould and soil flies. Newly emerged hoverfly adults were then collected daily for experiment or transfer to the main adult rearing cage.

Generation time was approximately 23 days, with 2 days as egg, 13 days as larva, and 8 days as pupa. This was slightly longer than that measured by Hart *et al.* (1997), probably due to the different species of aphid used by those authors.

2.2.5 Occurrence of parasitoids

Aphidophagous hoverflies are parasitized by several species of parasitic wasp, mainly of the Diplazontinae (Gilbert, 1993). Adult parasites are attracted to aphid colonies, where females actively search for hoverfly larvae and/or eggs, into which parasite eggs are laid. During culture of the hoverflies for this study, at least one parasitic wasp species was encountered, recurring on a number of occasions, and, initially, it was possible to lose large numbers of pupae to parasitism. For this reason it was necessary to check all cages daily for the presence of the wasps, which, when they occurred, could be observed flying against the top of the cage. When a parasite infection occurred, all hoverfly pupae were removed to a different location, in which fly (or parasite) emergence could take place in segregation. Any plants carrying hoverfly eggs that were exposed to the wasps were removed from culture. Any cages in which wasps were found were emptied and

thoroughly cleaned. It was also possible to identify some infected pupae by examination under a microscope, when parasite larvae were often visible under the surface of the pupal case. These steps were ultimately found sufficient to identify and limit parasitic wasp infection.

2.3 Quantification of insect cold hardiness

2.3.1 Measurement of supercooling point (SCP)

The SCP is the temperature at which spontaneous nucleation of body water occurs when an insect is cooled. Individual SCPs were determined by cooling from 20°C at 1°C min^{-1} in either a programmable refrigerated bath or Peltier cooling block. An attached Type T thermocouple monitored the individual's body temperature, providing readout via a data logger (Picolog® TC08) and PC. On freezing, a small release of energy is registered as a brief increase in body temperature, or exotherm, generally in the range 0.5-2.0°C, dependent on insect body mass. The body temperature immediately prior to the exotherm was recorded as the SCP.

2.3.2 Cold hardening treatments

The cold-hardening treatments employed in the following chapters involved holding individuals at various temperatures for various durations, or cooling/warming at various rates. Such treatments were applied in programmable refrigerated baths, accurate to ±0.3°C. These contained a 50% aqueous solution of ethylene glycol (car engine antifreeze). Mobile insects were held in plastic Beem® capsules of various sizes. These

were found preferable to Eppendorf® capsules, being less tapered and of much thinner (less insulating) plastic. In most experiments, only one insect was placed in a capsule, to avoid cross-inoculation of freezing. Samples were then placed in glass boiling tubes, plugged with cotton wool, for immersion in the bath.

Pupae were removed from plant stems and placed directly into boiling tubes; handling controls showed this had no deleterious effect on adult eclosion. Eggs were prepared for experiment by cutting away sections of leaf to which eggs were attached, and allowing the leaf sections to wilt slightly before fixing them with petroleum jelly to 10x100mm Perspex slides. These slides could then be inserted directly into glass boiling tubes. This method allowed the eggs to be manipulated without damage.

To monitor the insect exposure temperature, a blank tube was set up with an independent thermocouple inside the appropriate capsule, or on the Perspex slide surface etc. To ensure exposure of the insect to the correct temperature it was necessary to adjust the bath programme to compensate for differences in thermal insulation of different combinations of boiling tube and plastic capsule.

2.3.3 Survival and recovery

To monitor survival following treatment, individuals were kept in a variety of containers. Eggs were left on their Perspex slides, and stored in a ventilated plastic box. When the larva hatches, an empty papery shell is left behind, and these are readily counted with the aid of a microscope. Larvae were kept in glass vials with ventilated plastic lids covered in fine muslin. An aphid-infested section of bean seedling was provided and changed daily

when counting survivors. A high level of cannibalism was initially noted in larvae, even when aphids were present, and it was found best to keep them singly in vials. In the natural state, the pupa is firmly attached to the plant stem, enabling the emerging adult to pull itself free during eclosion. It was therefore found necessary to fix pupae in place following treatment. This was achieved by fixing the underside of the pupa to a square of filter paper with BluTack ®. Rows of such pupae were then stored in 6x10cm plastic boxes with mesh lids. Adult survival was monitored by keeping them in 15cm high x 8cm diameter clear plastic containers (in fact, plastic pint-glasses), with mesh-covered ventilation around the sides and a petri-dish lid. Pollen and sugar was provided in a small plastic box, and water on a cotton wool ball, in the base of the container. With daily water renewal, control adults were able to survive in these containers for a week with minimal mortality.

Mortality due to chilling injury may not occur for some time after exposure (Baust and Rojas, 1982). For this reason, most studies record survival after 72h. During this project however, it was observed that, after certain less severe treatments, many hoverfly larvae appeared to suspend development for much longer, up to a week, at which point they either died or continued to grow again. For this reason, survival was recorded for larvae at 72h or, if longer, at progression to the next stage (i.e. moulting to the next larval instar, or pupation). In eggs, survival was recorded as the number of successfully hatched first instar larvae. In pupa, complete eclosion of the adult was recorded. Adult survival was recorded at 72h; difficulty in ensuring mating meant that it was not possible to record onset of reproduction.

2.3.4 Basic design of rapid cold-hardening (RCH) experiments

The protocol used for identifying RCH followed that established in early studies on other species (e.g. Lee *et al.*, 1987; Czajka & Lee, 1990; Coulson & Bale, 1992). Samples were first transferred directly from the rearing temperature to a range of low exposure temperatures for a set duration ('direct transfer exposure'), and the response recorded (survival, chill coma temperature, flight etc). An exposure eliciting a response level of approximately 25% was then selected: the so-called 'discriminating temperature'. Samples were then hardened under various regimes, before exposure at the discriminating temperature. An RCH response is characterised by an increase in hardiness over the direct transfer level. This procedure is illustrated in Figure 2.1.

While temperature changes as abrupt as those involved in such direct transfer exposures may be uncommon in nature, they are not entirely without basis. For example, leaves in forest canopies experience rapid light fluctuations, termed 'sunflecks', which occur frequently throughout the day due to gaps between clouds, leaf flutter and canopy sway in windy conditions (Pearcy, 1990). These variations in incident solar radiation can rapidly change leaf temperature. For example, Singsaas and Sharkey (1998) measured leaf surface temperature at the top of a 30m tall oak *Quercus alba* in North Carolina, USA. Changes in insolation resulted in rapid temperature fluctuations throughout the day, generally of the order of 1-3°C over a few minutes, but temperature changes of more than 8°C occurred regularly, lasting between 15 sec and 20min.

Insects in such an environment could thus also experience temperature changes equivalent to 1°C min^{-1} or faster, as used in direct transfer RCH experiments. However, various

studies have considered much slower cooling rates, typically 0.1 to 0.05°C min^{-1}, to be more ecologically realistic, and have shown that RCH also occurs during both slow cooling (McDonald *et al.*, 1997; Kelty and Lee, 1999; Broufas and Koveos, 2001; Powell and Bale, 2004), and 'realistic', cycling thermoperiods in the laboratory (Kim and Song, 2000; Kelty and Lee, 2001) and in field-sampled individuals at different times of day (Koveos, 2001).

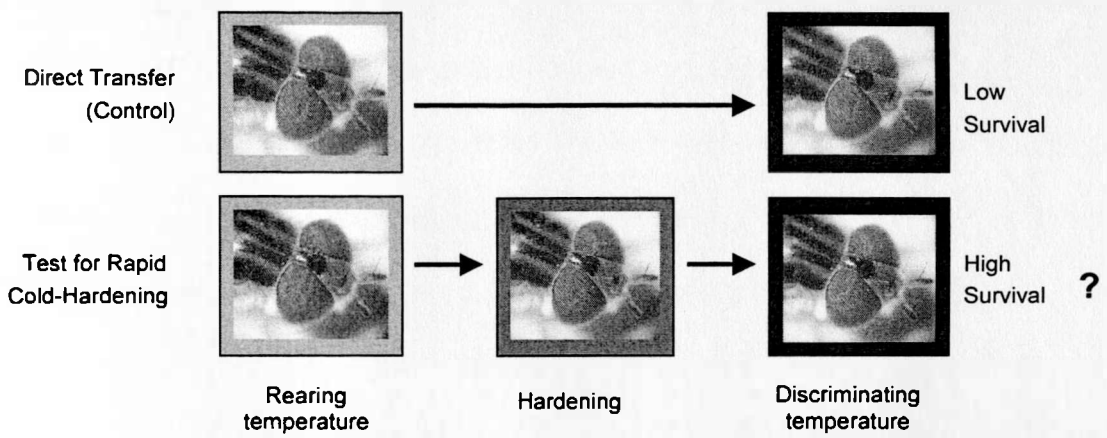

Figure 2.1 Protocol to quantify the rapid cold-hardening response in an insect. While most studies examine the effect of RCH on survival at lethal temperatures, the protocol can also be applied to other measures of cold hardiness.

Chapter 3

Relation of rapid cold-hardening ability to supercooling point and lower lethal temperature in the life cycle stages of *Episyrphus balteatus*

3.1 Introduction

Rapid cold-hardening (RCH) is a process whereby the survival of insects at sub-zero temperatures is increased if the subject is first acclimated for a short period at a less extreme temperature (Lee *et al.*, 1987). The process has been demonstrated in a wide range of species, and is thought to protect against cold shock injury and allow an insects' physiology to track daily changes in environmental temperature, 'fine-tuning' their response (Czajka & Lee, 1990; Wang *et al.*, 2003; Shreve *et al.*, 2004). However, most studies of RCH have focused on individual life cycle stages of the species investigated, although cold hardiness in general is known to vary between life stages.

In most species in which RCH has been studied, the range of temperatures in which mortality occurs due to chilling injury, described as the lower lethal temperature (LLT), lies well above the freezing temperature (or supercooling point, SCP), and RCH operates to depress the LLT by reducing mortality due to chilling injury. For example, in pupae of the housefly *Musca domestica*, the SCP was in the range -16 to -22°C (Coulson and Bale, 1990). However, no individuals survived 2h at -7°C, and mortality at this temperature was therefore due to chilling injury (the LLT). RCH took place during 15 to 90min exposures at 0°C, greatly increasing subsequent survival at -7°C. This is the classic RCH response.

A notable exception to this general process occurs in three species of Antarctic arthropod: the collembolon *Cryptopygus antarcticus*, and the mites *Alaskozetes antarcticus* and *Halozetes belgicae* (Worland *et al.*, 2000; Worland and Convey, 2001). These species are freeze avoiding, and develop seasonal cold hardiness in preparation for the Antarctic winter. However, in summer-acclimated individuals, RCH increases cold hardiness by lowering the SCP. The process does not appear to be associated with gut clearance, and so, during the Antarctic summer, allows the SCP of individuals in the field to vary with microhabitat temperature, providing cold hardiness without necessitating entry into an inactive state.

In these species, the SCP is not an immoveable lower limit to the cold hardiness of individuals. However, in most freeze avoiding species, RCH acts to reduce mortality due to chilling injury that occurs well above the freezing point (Bale, 2002; Wang *et al.*, 2003).

Variation in SCP and LLT through the life cycle has been investigated in a number of species. Coulson and Bale (1990) measured SCP in *Musca domestica*. This was lowest in the egg (-27°C), increased rapidly through the larval stages (to a maximum of -6°C), declined again on pupation (-22°C), increased toward the end of the pupal phase (-16°C) and was higher in the adult (-14°C). This variation may be explained by increased presence of ice nucleators in the gut of feeding stages, which are egested and/or masked in non-feeding stages (Duman, 2001).

Variation in SCP may also be linked, in part, to individual body mass at each stage. A positive relationship between SCP and body mass in ectotherms, with smaller individuals having lower SCPs, has been identified by a number of studies, both between species of

diverse taxa (Lee and Constanzo, 1998; Waller *et al.*, 2006), and within individuals of the same species (Pugh, 1994; Johnston and Lee, 1990). Waller *et al.* (2006) investigated 11 Antarctic intertidal invertebrate species (from 9 classes) ranging in size over 5 orders of magnitude, and found a significant positive relationship between mean species body mass and SCP. Johnston and Lee (1990) also found a significant positive correlation between body mass and SCP in individuals of the beetle *Tenebrio molitor*.

Greater mass may result in higher SCP because, as body mass increases, the larger volume of body water means that (1) the probability of spontaneous ice-nucleation increases, and (2) the probability of an active ice-nucleator being present is also higher (Lee and Constanzo, 1998). Smaller water droplets supercool to lower temperatures (Bigg, 1953), and it has been suggested that supercooled Antarctic mites behave in the same way, with smaller individuals having lower SCPs (Rotheray and Block, 1992). Pugh (1994) also related lower body-water content in small mites to decreased probability of freezing at higher sub-zero temperatures. However, Bauer *et al.* (2001) found that, while cocoons of the potworm *Enchytreaus crypticus* had both greater mass and higher SCP when reared on agar with nettles, as opposed to agar with oats, body water content was not significantly different. It was therefore concluded that probable differences in the ice-nucleating bacteria ingested in the two diets were the likely cause of the difference in SCP (Bauer *et al.*, 2001).

Not all studies, however, have identified a positive relationship between mass and SCP. Indeed, in some partially freeze-tolerant molluscs, such as the land snail *Cornu aspersum*, older (larger) individuals are more cold-hardy than immatures, having a lower SCP and being able to tolerate a greater degree of ice formation (Ansart and Vernon, 2004). Block *et al.*

(1990) measured SCP in all life stages of two blowfly species, *Calliphora vicina* and *C. vomitoria*, and found considerable variation, but this did not correlate with the amount of body water (nor the concentration of a range of solutes). There was also no significant correlation between body mass and SCP in the termite *Reticulitermes flavipes* (Cabrera and Kamble, 2004). Thus, while a general relationship between mass and SCP is recognised (Lee and Constanzo, 1998), there is conflicting evidence, and few studies have attempted to model this relationship (Waller *et al.*, 2006).

In addition to the SCP, the LLT can also vary significantly between life stages. This has been shown in, for example, larvae, nymphs and adults of the tick *Ixodes scapularis* (Vandyk *et al.*, 1996). The relationship between SCP and LLT is not necessarily constant. For example, Rosales *et al.* (1994) found no correlation between SCP and survival at low temperatures in all stages of *Musca autumnalis* and *M. domestica.*

RCH has also been examined in different life stages of the same species. RCH has been demonstrated in both early pupae and pharate adults of *Sarcophaga bullata*, and in larvae, pupae and pharate adults of *S. crassipalpis*, with significant effects in each of the stages (Lee *et al.*, 1987; Chen *et al.*, 1987). Similar results were obtained for larvae, pupae and adults of *Drosophila melanogaster* (Czajka and Lee, 1990). Chen *et al.* (1991) measured the effect of RCH on survival of cold shock in larvae, pupae and adults of the flesh fly *Sarcophaga crassipalpis*. Hardening increased the duration of survival at -10°C in all stages, but the effect was significantly greater in the pupa. This was also the case in survival of heat shock at 45°C, following hardening at 40°C.

Powell and Bale (2004) found that RCH capacity was higher in nymphs than in adults of the grain aphid *Sitobion avenae*, and suggested that higher overwintering survival in nymphs may be of more benefit to the species than in adults which may be close to completing their reproductive output. RCH capacity has also been shown to vary with age within life-stages. In *Drosophila melanogaster*, 1d-old flies were less able to rapidly cold-harden than 3-5d old flies, and the effect then declined with age to 30 days (Czajka and Lee, 1990). Adult SCP remained fairly constant with age.

Unlike life-stage and age differences in cold-hardiness or RCH ability, most studies have not found sex differences in adults. For example, there was no difference in LT_{50} (LLT at which 50% mortality occurs) between male and female ticks *Ixodes scapularis* (Vandyk *et al.*, 1996). RCH was equally effective in adult male and female *Drosophila melanogaster* (Czajka and Lee, 1990) and *Bactracea oleae* (Koveos, 2001).

Cold tolerance and RCH have not been extensively researched in hoverflies (Diptera: Syrphidae). *Episyrphus balteatus* is a common hoverfly in the UK. The larvae are aphidophagous and are an important natural enemy of aphids, including many pest species (Chambers & Adams, 1986). In the parts of its range where the species overwinters, the overwintering morph is thought to be the mated female (Schneider, 1969). However, *E. balteatus* does not overwinter in the UK in significant numbers, but adults migrate to warmer regions in southern Europe during the autumn, with northward migration in the spring (Ball and Morris, 2000). Hart & Bale (1997c) showed that the adult is freeze avoiding, and that overall cold hardiness is weak, with only 10% survival after 10d at temperatures between 5 and -5°C. It is thought that this low level of cold hardiness contributes to this species' need to

avoid the UK winter by migration. However, the cold hardiness of the life stages preceding the adult has not been investigated.

Given that SCP varies greatly through the insect life cycle, particularly between feeding and non-feeding stages, it can be hypothesised that RCH ability would be affected by the changing relationship between LLT and SCP. This study examined this relationship, comparing the SCP, LLT and RCH in each life cycle stage of *E. balteatus*. From an extensive literature search, it would appear that this is the first attempt to characterise the relative changes in these three parameters in all life stages of a single species.

3.2 Aims

- Determine the freezing temperature (SCP), lower lethal temperature (LLT) and capacity for rapid-cold hardening (RCH) in each the six life stages of *E. balteatus*: the egg, three larval instars (L1, L2 and L3), the pupa, and the adult (both male and female).
- Test the hypothesis that SCP is influenced by individual body mass, by rearing artificially underweight individuals.
- Identify the limits placed on RCH by SCP and LLT.

3.3 Methods

Eggs of *E. balteatus* were collected from a laboratory culture in 24h batches and reared on to provide individuals of the required life stage. Larvae to be used in experiments were fed on only one species of aphid, *Aphis fabae*, to help standardise developmental rate. Larvae and adults were actively feeding when treated (gut contents visible). Samples of each of the six life stages were used for experiments on the following days after egg collection:

1) Egg: day 1
2) L1: day 3
3) L2: day 6
4) L3: day 8
5) Pupa: 5d post-pupation
6) Adult: 1d post-eclosion

The timings for pupae and adults were defined as post-pupation and post-eclosion respectively because of slight variation in development rate between individuals within batches. This became evident at the point of pupation, which was spread over 1-3d. New adults emerged over a 2-5d period.

3.3.1 Supercooling Point (SCP)

Samples of 28 to 32 individuals were cooled in a refrigerated bath at 1°C min^{-1} from 20°C to the SCP. An attached thermocouple recorded the freezing exotherm via a data logger and PC.

While it was possible to remove pupae from leaves and stems without damaging them, removal of eggs caused the outer papery case to be ripped. In order to determine the SCP of eggs *in situ* on the leaf surface, and distinguish it from the SCP of the leaf tissue, *in situ* eggs were cooled and both SCPs recorded. These were then compared to SCPs for eggs removed from the leaf surface, and SCPs for samples of leaf tissue only.

SCPs were determined for each of the 7 life stages listed above, plus: (1) the L3 on day 6, the same day used for the L2; and (2) the L3 after gut evacuation (this occurred on day 15 ±1d; there were only a few hours between gut evacuation and pupation, during which time the SCP was recorded). Survival after freezing was recorded at 24h intervals for 3d. Mean SCPs were analysed with ANOVA and Tukey's multiple pairwise comparisons.

In order to test the hypothesis that body mass affects SCP, adult mass was recorded along with SCP. All life stages were reared by providing larvae with excess aphid prey, and the resulting adults had approximately uniform mass. Therefore, to introduce greater variation in adult mass, a further group of underweight adults were reared. This was done by limiting the food available to larvae. Batches of eggs were collected and allowed to develop on a set number of aphid-infested bean-seedling trays. By trial and error, the number of trays on which approximately half of the larvae would reach pupation was identified. The resulting pupae were deemed to be close to the minimum threshold weight for successful pupation. The other half of the larvae died through starvation. The mass and SCP of the underweight

pupae were then recorded. The relationship between SCP and mass was then modelled by simple linear regression.

3.3.2 Lower Lethal Temperature (LLT)

Samples of each life stage were cooled at 1°C min^{-1} from 20°C to the exposure temperature, held for 1min, and then warmed to 20°C at 1°C min^{-1}. This was carried out for a range of sub-zero exposure temperatures. Survival was recorded at 24h intervals, for 3d or until the whole sample had successfully progressed to the next life stage, whichever was the longer.

Probit analysis was then used to determine the LLT. This procedure performs probit transformation to linearise the sigmoid dosage response curve of the survival data, and regression to determine dosage percentiles (Finney, 1971). Three indices of lethal temperature were thus determined: LT_{10}, LT_{50} and LT_{90}, the temperatures resulting in 10, 50 and 90% mortality respectively.

3.3.3 Rapid cold-hardening (RCH)

To determine the discriminating temperature, samples were placed at a range of sub-zero temperatures for 2h. Survival of these direct transfer treatments was recorded at 24h intervals, for 3d or until the sample had progressed to the next life stage, whichever was the longer. From these data, the exposure which yielded approximately 25% survival was selected as the discriminating temperature, at which to test for RCH. Samples were then hardened at 0°C for 10, 20, 40, 60 and 80min (plus 120min in adults), before exposure at the discriminating

temperature. Sample sizes varied according to the availability of each stage, and were as follows: Egg: 5 replicates of 50; Larvae: 6 replicates of 10; Pupa, 5 replicates of 10; Adults, 6 replicates of 8. For each life cycle stage, all treatments (for discriminating temperature and test for RCH) were carried out in succession, such that the individuals used were of the same cohort collected from the stock culture over the minimum period practical. The direct-transfer exposure to the discriminating temperature was therefore not repeated, but, for clarity, the result is presented twice, once on each of the graphs in the pairs that follow (e.g. '-12' on Figure 3.5 and 'DT' on Figure 3.6 represent the same dataset).

Mean survival of the discriminating temperature was then compared in hardened and non-hardened groups by ANOVA and Tukey's multiple comparisons. RCH is identified as an increase in survival in hardened individuals over that of the direct transfer sample.

3.4 Results

3.4.1 Change in SCP through the life cycle

The SCP of undisturbed eggs was identified by cooling eggs *in situ* on the leaf. Two exotherms were observed. The mean SCP of the upper exotherm was -11.7 ± 0.6°C, and that of the lower was -25.3 ± 0.2°C. The SCPs of leaf tissue only and egg only (detached from the leaf) corresponded ($p<0.01$) to the upper and lower exotherms of the *in situ* egg samples (see Table 3.1). This identified the lower exotherm as that of the egg, and suggested that, at least

in terms of SCP, the egg was not damaged by removal from the leaf. However, only the data for *in situ* eggs were used for further analysis.

Table 3.1 Identification of *in situ* egg SCP in *Episyrphus balteatus*: comparing upper and lower exotherms of egg on leaf with leaf-only and egg-only SCPs. Means followed by the same letter are not significantly different at p = 0.05.

	Mean ± SEM (°C)	
Egg on leaf: upper exotherm	-11.7 ±0.64	a
Egg on leaf: lower exotherm	-25.3 ±0.24	b
Leaf only	-13.3 ±0.53	a
Egg only	-25.5 ±0.15	b

The SCPs of the life-cycle stages examined are presented in Table 3.2. There were significant differences between stages. The SCP of the egg was the lowest (-25.3 ± 0.2°C). This increased significantly in the first larval instar (-17.2 ± 1.0°C) and again in the second (-7.4 ± 0.4°C). There was a further slight but non-significant increase in the third larval instar (-6.0 ± 0.1°C); this was the stage with the highest SCP. There was no difference ($p>0.05$) between the second and third instars on the same day, i.e. immediately before and after the moult from second to third. The SCP of the third instar decreased by approximately 1°C following gut evacuation, but this was not statistically significant ($p>0.05$). In the pupa however, the SCP was significantly lower (-16.6 ± 1.0°C), similar to that of the first larval instar. The SCP of adults was significantly higher again. Adult females had a lower SCP (-8.9 ± 0.5°C) than males (-7.0 ± 0.1°C), but not significantly so. These changes are presented relative to time in Figure 3.1.

Table 3.2 Mean ± SEM and range of SCP of the life stages of *Episyrphus balteatus*. L1/2/3 are first/second/third larval instar. Means followed by the same letter are not significantly different at p = 0.05.

	Mean ± SEM (°C)		Range (°C)
Egg	-25.3 ± 0.3	a	-19.8 to -27.2
L1	-17.2 ± 1.2	b	-6.1 to -26.4
L2	-7.4 ± 0.4	c	-5.8 to -17.2
L3 (day 6)	-7.1 ± 0.3	c	-5.9 to -13.0
L3 (day 8)	-6.0 ± 0.1	c	-4.4 to -6.8
Gut-evacuated L3	-7.0 ± 0.5	c	-2.3 to -11.1
Pupa	-16.6 ± 1.0	b	-5.1 to -22.2
Adult Male	-7.0 ± 0.1	c	-6.1 to -8.2
Adult Female	-8.9 ± 0.5	c	-5.7 to -15.2

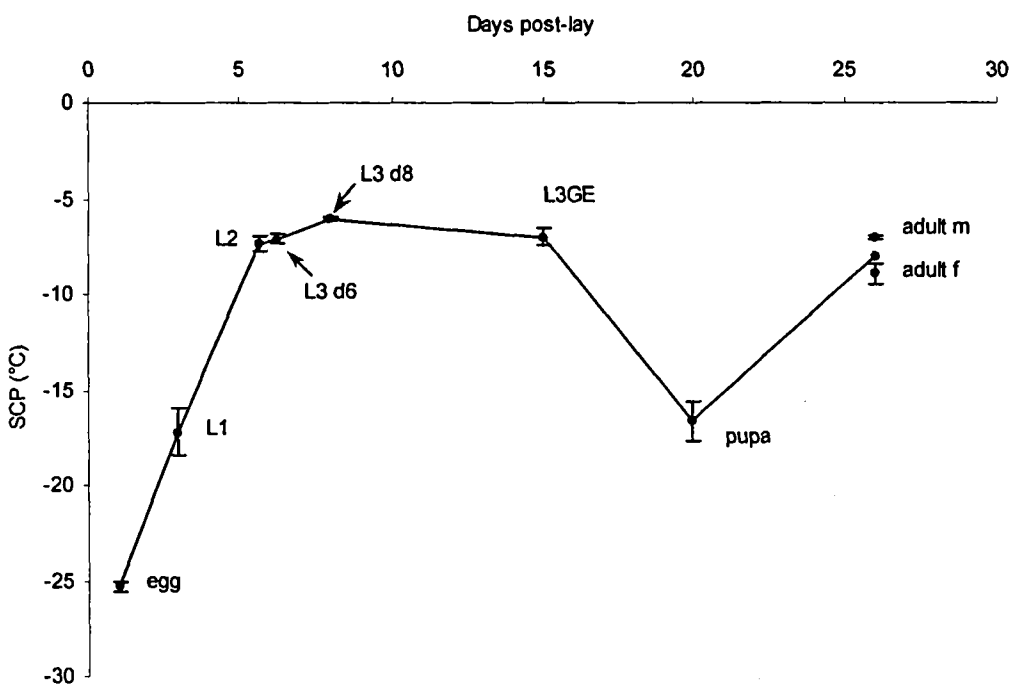

Figure 3.1 Change in SCP through the life cycle of *Episyrphus balteatus*. Error bars show SEM. L1 = first instar larva. L2 = second instar larva. L3 d6 = third instar larva, day 6. L3 d8 = third instar larva, day 8. L3GE = gut evacuated third instar larva. Adult m = male, f = female.

There was also considerable variation between individuals within certain stages, as evidenced by the SCP ranges shown in Table 3.2. With the exception of the egg, the range of SCPs was particularly broad in stages with low means: the first and second larval instars and the pupa. No individuals survived freezing. The SCP distribution for each life stage is shown in Figure 3.2. While the distributions for each stage were unimodal, there were also a number of outliers in each case, and the dispersion was particularly high in the first larval instar.

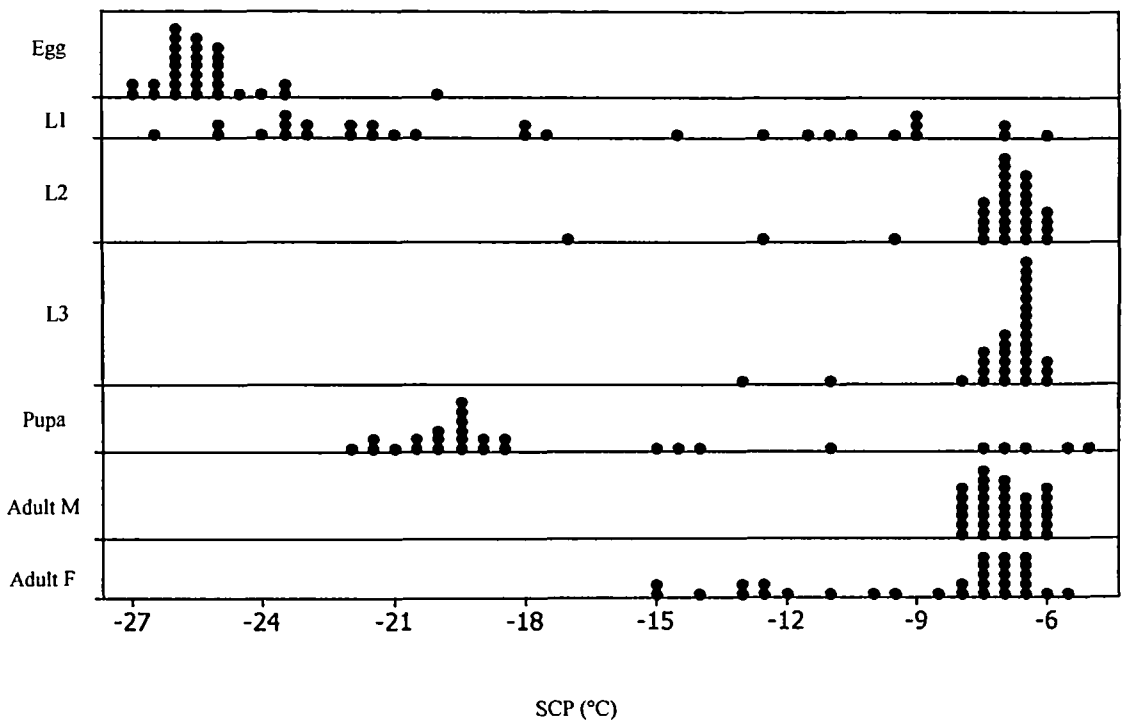

Figure 3.2 Distribution of SCP in each life stage of *Episyrphus balteatus*. L1/2/3 = first/second/third larval instar. Each point represents one individual.

3.4.2 SCP and adult mass

The mean SCPs and masses of males and females in the normal weight and underweight groups are presented in Table 3.3. While SCP was similar in both female weight groups, SCP was lower in underweight than in normal weight males. In order to test for a relationship between mass and SCP, the weight groups were pooled for each sex and a linear regression model was fitted. In females, there was no significant correlation between mass and SCP ($p=0.6$); in males, there was a significant relationship, but only a small proportion of the variation in SCP was explained by mass ($p<0.01$, $R^2 = 11\%$).

Table 3.3 Mean mass and SCP in normal weight and underweight male and female *Episyrphus balteatus*.

	Mean Mass ± SEM (mg)	Mean SCP ± SEM (°C)
Normal weight male	23.3 ± 0.5	-7.0 ± 0.1
Normal weight female	19.0 ± 0.6	-8.9 ± 0.5
Underweight male	14.4 ± 0.4	-8.0 ± 0.4
Underweight female	11.7 ± 0.7	-8.8 ± 0.6

3.4.3 Lower lethal temperature (LLT)

Lower lethal temperature was examined in the six principal life stages. The temperatures that resulted in 10% (LT_{10}), 50% (LT_{50}) and 90% (LT_{90}) mortality are presented in Figure 3.3. The change in LLT from stage to stage was broadly similar to that of SCP, and was similar at each mortality level measured. In adults, the LT_{10} was slightly higher in males than in

females (-3.9°C vs. -5.6°C), and this difference was no longer evident at LT_{90}, i.e. mortality in males was spread over a slightly broader range of temperatures than in females. The male-female difference was not, however, significant (indicated by overlapping 95% fiducial limits).

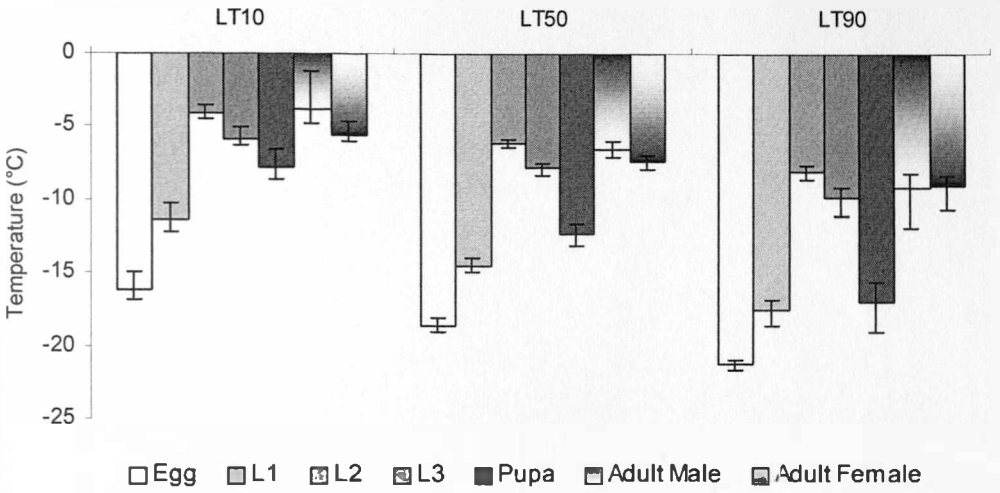

Figure 3.3 Lower lethal temperatures of the life cycle stages of *Episyrphus balteatus*. Temperatures causing 10%, 50% and 90% mortality (LT10, 50 and 90 respectively). Error bars show 95% fiducial limits.

The relationship between SCP and LLT is shown in Figure 3.4. The only life stage in which the SCP was significantly below the LLT range was the egg. In the first and second larval instars, the pupa, and the adult female, the SCP was close to the LT_{90}. In the third instar larva and the adult male, the SCP was similar to the LT_{50}.

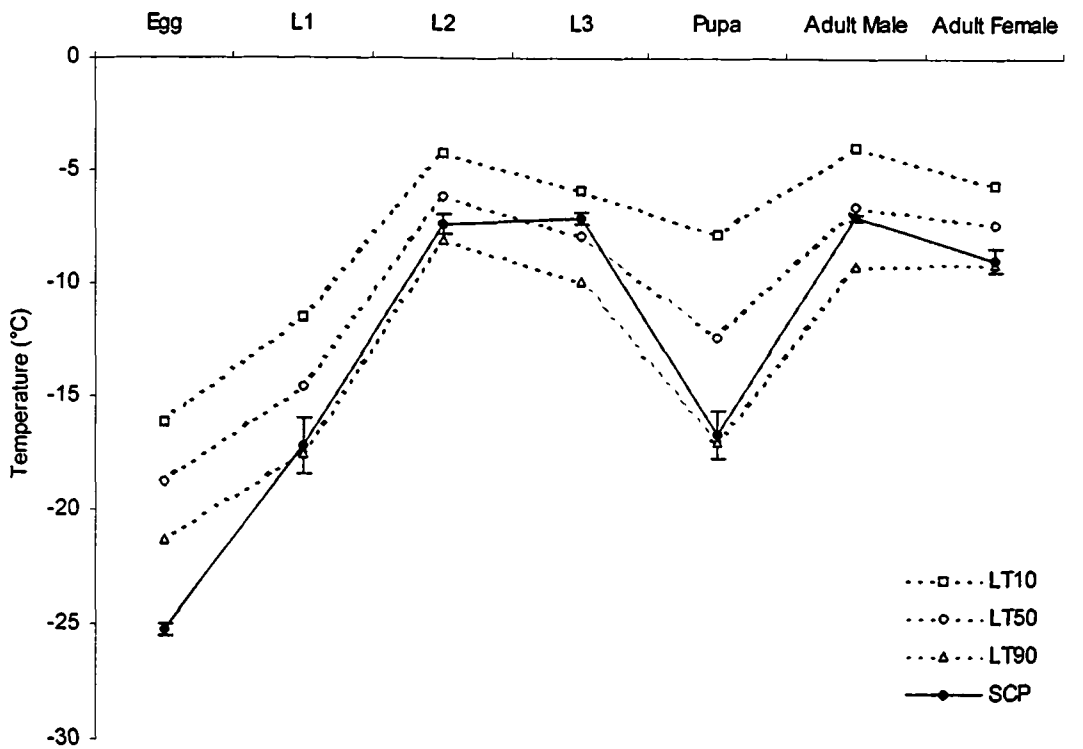

Figure 3.4 Variation in SCP relative to LLT in each life stage of *Episyrphus balteatus*. Horizontal axis is categorical: lines are included above only to aid visualisation of LLT range and SCP changes through life cycle. Error bars show SEM. L1/2/3 = first/second/third larval instar. LT10/50/90 = Temperature resulting in 10/50/90% mortality.

3.4.4 Rapid cold-hardening (RCH)

Egg. Only 50% of control eggs hatched successfully. After direct transfer to -6°C, 25 ± 4.2% survived, and this figure fell steadily with decreasing temperature, reaching zero at -14°C (Figure 3.5). Based on these data, -12°C (survival = 11 ± 3.3%) was selected as the discriminating temperature at which to test for RCH.

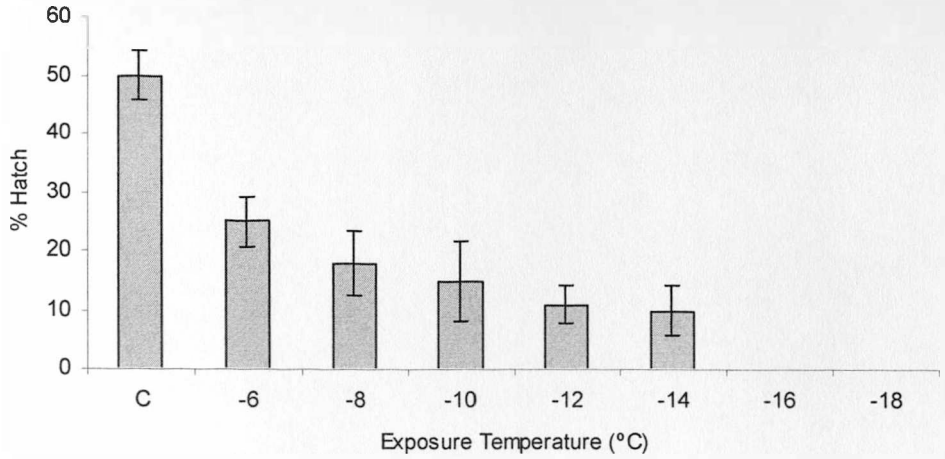

Figure 3.5 Survival (% hatch rate) of eggs of *Episyrphus balteatus* after 2h exposures at -6 to -18°C. C = control. Error bars show SEM.

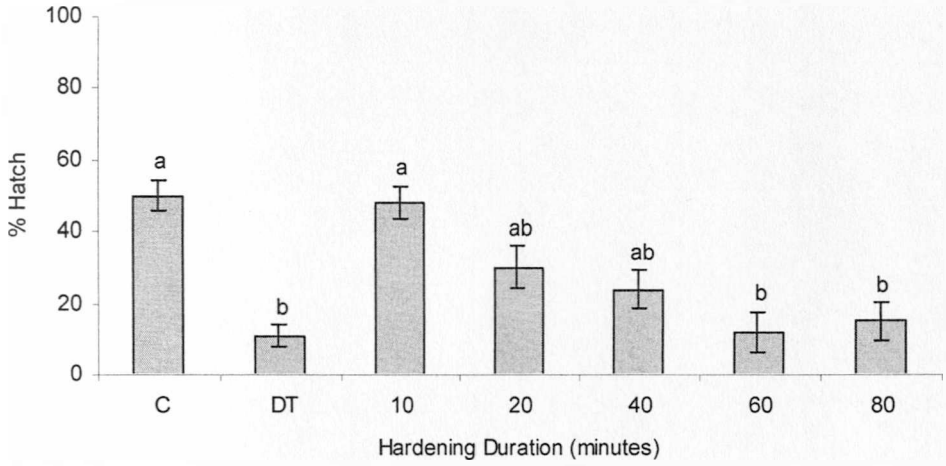

Figure 3.6 RCH in eggs of *Episyrphus balteatus*: survival of -12°C (2h) following hardening at 0°C for 10 to 80 min. Error bars show SEM. C = Control. DT = survival following direct transfer to -12°C (2h) without hardening. Means shown with the same letter are not different at p = 0.05.

The effect of hardening treatments on survival of this exposure is shown in Figure 3.6. Following hardening at 0°C for just 10min, survival of the discriminating temperature increased significantly by 37% to 48 ± 4.6% ($p<0.05$), a rate similar to the control. With increasing duration of hardening beyond 10min, survival declined quickly to the direct transfer rate.

First instar larva. Survival in the control group was 88 ± 3.1%. The effect of direct transfer to temperatures from 0 to -10°C for 2h is shown in Figure 3.7. Survival declined steadily from 0 to -6°C, and then more quickly to zero at -10°C. The exposure to -9°C (survival = 25 ± 6.2%) was selected as the discriminating temperature. The effect of hardening is shown in Figure 3.8. Hardening for 10 to 60min increased survival significantly ($p<0.05$). Again, hardening for 10min induced the greatest increase in survival, to a level not significantly different from the control, and the trend declined gradually with increasing duration of hardening.

Second instar larva. Survival in the control was 93 ± 2.1%. The effect of the direct transfer exposures is shown in Figure 3.9. Survival was high at -4 and -5°C, and fell rapidly to zero at -9°C. The -6°C exposure (survival = 30 ± 19%) was selected as the discriminating temperature. The effect of hardening on survival of this exposure is presented in Figure 3.10. All hardening durations increased survival to the control level, although only the optimal hardening duration of 20min induced a significant increase over the direct transfer rate ($p<0.05$).

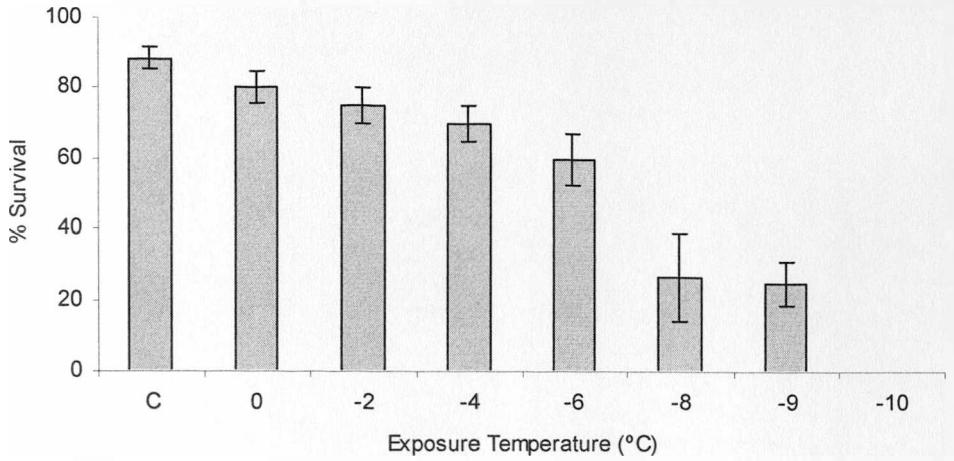

Figure 3.7 Survival of first instar larvae of *Episyrphus balteatus* after 2h exposures at 0 to -10°C. C = control. Error bars show SEM.

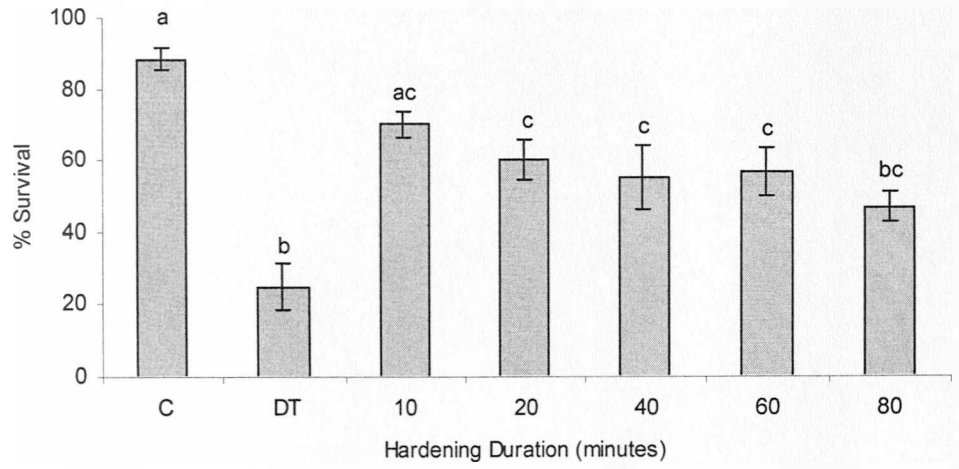

Figure 3.8 RCH in first instar larvae of *Episyrphus balteatus*: survival of -9°C (2h) following hardening at 0°C for 10 to 80 min. Error bars show SEM. C = Control. DT = survival following direct transfer to -9°C (2h) without hardening. Means shown with the same letter are not different at p = 0.05.

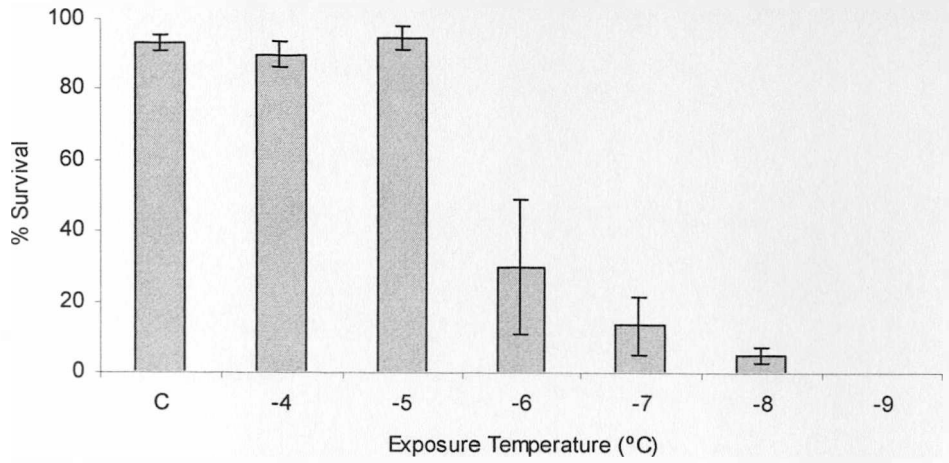

Figure 3.9 Survival of second instar larvae of *Episyrphus balteatus* after 2h exposures at -4 to -9°C. C = control. Error bars show SEM.

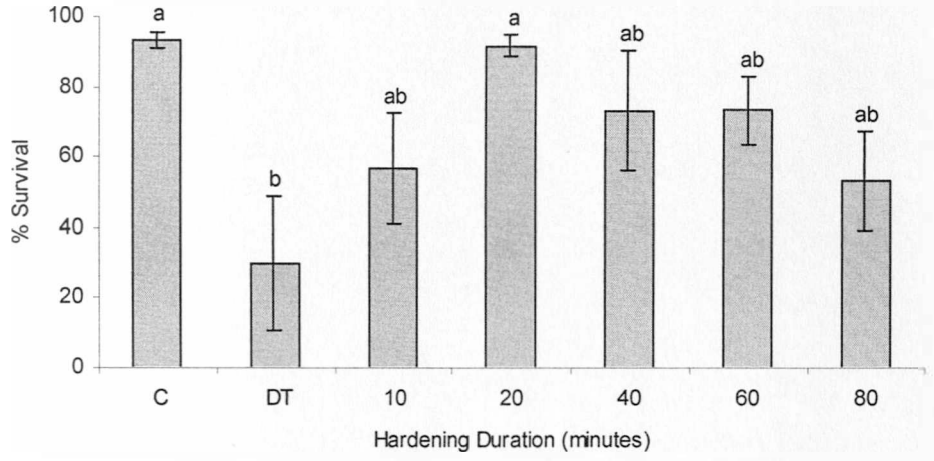

Figure 3.10 RCH in second instar larvae of *Episyrphus balteatus*: survival of -6°C (2h) following hardening at 0°C for 10 to 80 minutes. Error bars show SEM. C = Control. DT = survival following direct transfer to -6°C (2h) without hardening. Means shown with the same letter are not different at p = 0.05.

Third instar larva. The control survival rate was 97 ± 3.0%. Survival of the direct transfer exposures was high at -3 and -4°C, but then fell very rapidly to zero at -6°C (Figure 3.11). The variance in survival of -5 and -5.5°C was very high. This meant that it was not possible to select a discriminating temperature that yielded statistically lower survival than the control. However, -5.5°C (survival = 45 ± 21%) was used as the discriminating temperature, and the effects of hardening are presented in Figure 3.12. There was great variation in the survival rates for each hardening duration, and although hardening for 20min did increase survival from 45% to 92%, 60min hardening decreased survival significantly, and there was no clear pattern in hardening effect.

Pupa. The control survival rate in pupae was 88 ± 4.9%. Survival of direct transfer exposures declined steadily from 84% at -2°C to zero at -9°C (Figure 3.13). At a discriminating temperature of -8°C (survival = 32 ± 5.8%), hardening increased mean survival at all durations (Figure 3.14). However, the shape of the response was unusual, and appeared to show some bimodality. Ten and 80min hardening increased survival significantly over the direct transfer rate ($p<0.05$), to levels equivalent to the control. The 40min hardening treatment increased survival from 32 ± 5.8% to 50 ± 5.5%, but this was not statistically significantly. The 20 and 60min hardening durations also increased survival, to more than 60%, but again these were not significantly different to the direct transfer level.

In many cases it is a feature of the results of this type of experiment that even the optimal RCH treatment does not induce a level of survival equal to that of the control. In order to investigate this effect in pupae, a further experiment was conducted, using a discriminating

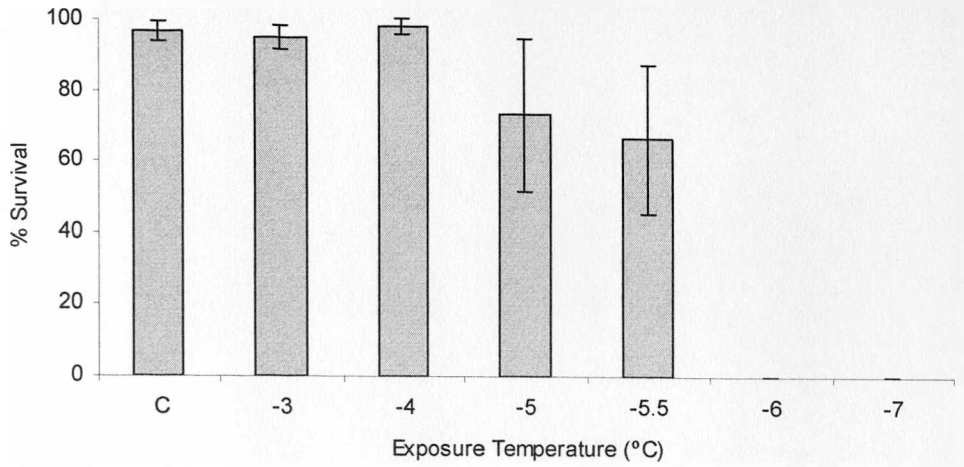

Figure 3.11 Survival of third instar larvae of *Episyrphus balteatus* after 2h exposures at -3 to -7°C. C = control. Error bars show SEM.

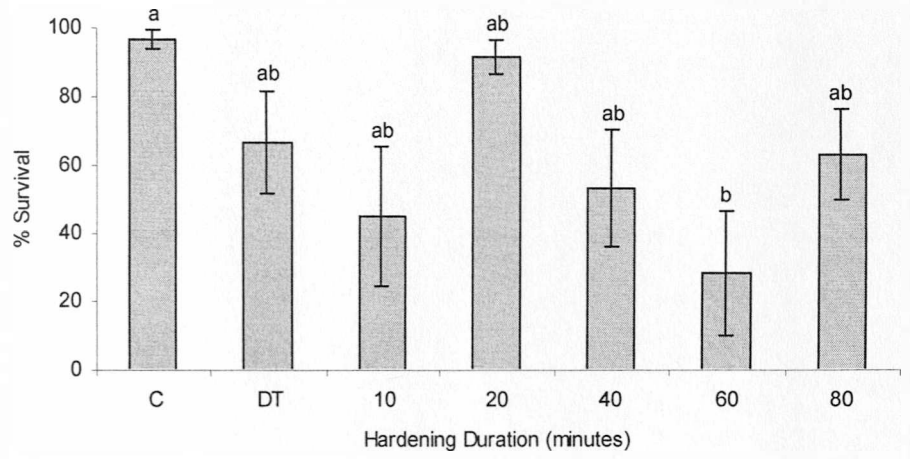

Figure 3.12 Effect of hardening in third instar larvae of *Episyrphus balteatus*: survival of -5.5°C (2h) following hardening at 0°C for 10 to 80 minutes. Error bars show SEM. C = Control. DT = survival following direct transfer to -5.5°C (2h) without hardening. Means shown with the same letter are not different at p=0.05.

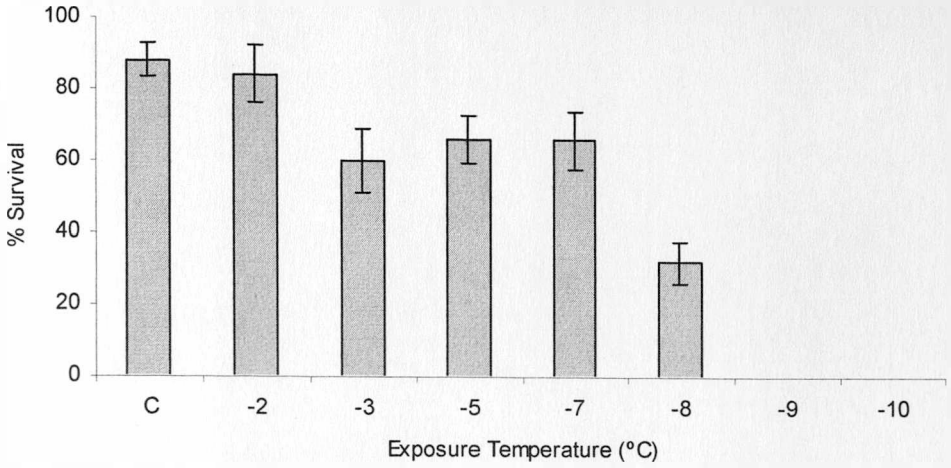

Figure 3.13 Survival in pupae of *Episyrphus balteatus* after 2h exposures at -2 to -10°C. C = control. Error bars show SEM.

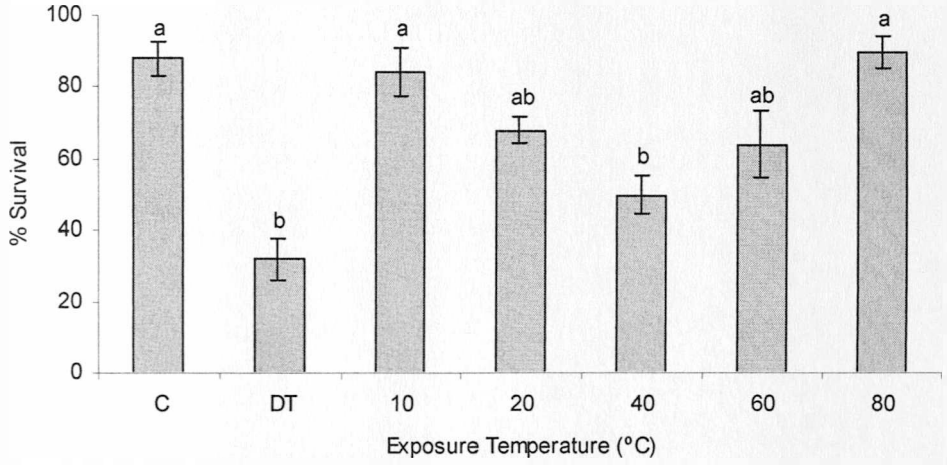

Figure 3.14 RCH in pupae of *Episyrphus balteatus*: survival of -8°C (2h) following hardening at 0°C for 10 to 80 minutes. Error bars show SEM. C = Control. DT = survival following direct transfer to -8°C (2h) without hardening. Means shown with the same letter are not different at p = 0.05.

temperature at which survival was much closer to that of the control. A second control was performed, with 98 ± 2% survival. At -7°C, 66 ± 8.1% of pupae survived direct transfer exposure. Hardening did increase mean survival, but this was not significant, and remained significantly below the control rate ($p<0.05$) (Figure 3.15).

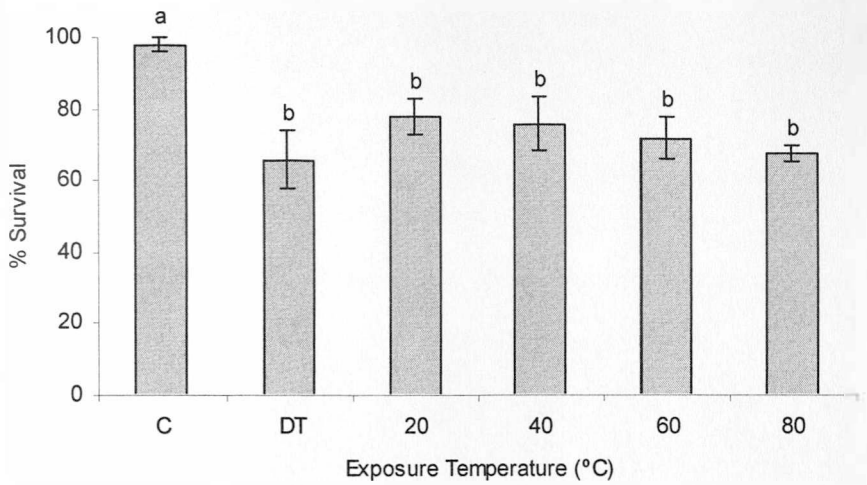

Figure 3.15 Effect of hardening treatments at a less stressful temperature in pupae of *Episyrphus balteatus*: survival of -7°C (2h) following hardening at 0°C for 10 to 80 minutes. Error bars show SEM. C = Control. DT = survival following direct transfer to -7°C (2h) without hardening. Means shown with the same letter are not different at p = 0.05.

Adult male. The control survival rate was 98 ± 2.6%. Direct transfer to -2 and -4°C did not decrease survival below this, after which survival declined steadily to zero at -10°C (Figure 3.16). The discriminating temperature was -8°C (survival = 15 ± 6.0%). Hardening for 10min increased survival to 35 ± 8.2%, but this was not statistically greater than the direct transfer rate, and increasing hardening duration actually decreased mean survival below the direct transfer rate (Figure 3.17).

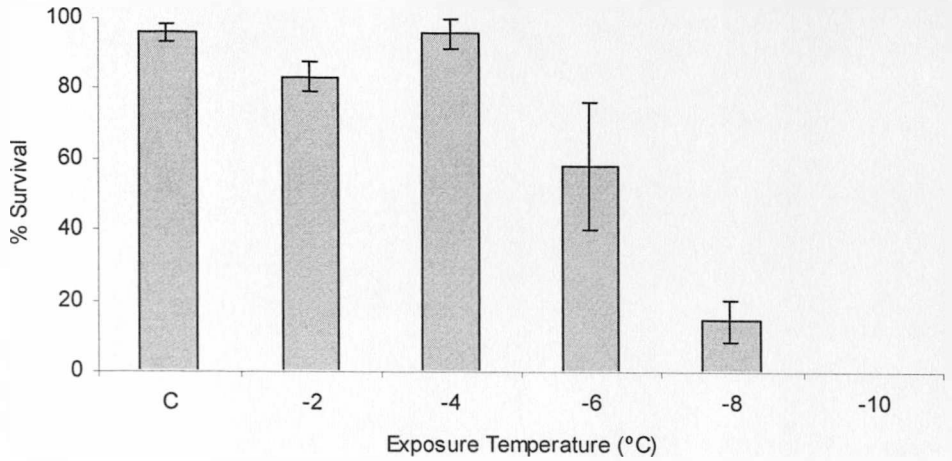

Figure 3.16 Survival of adult male *Episyrphus balteatus* after 2h exposures at -2 to -10°C. C = control. Error bars show SEM.

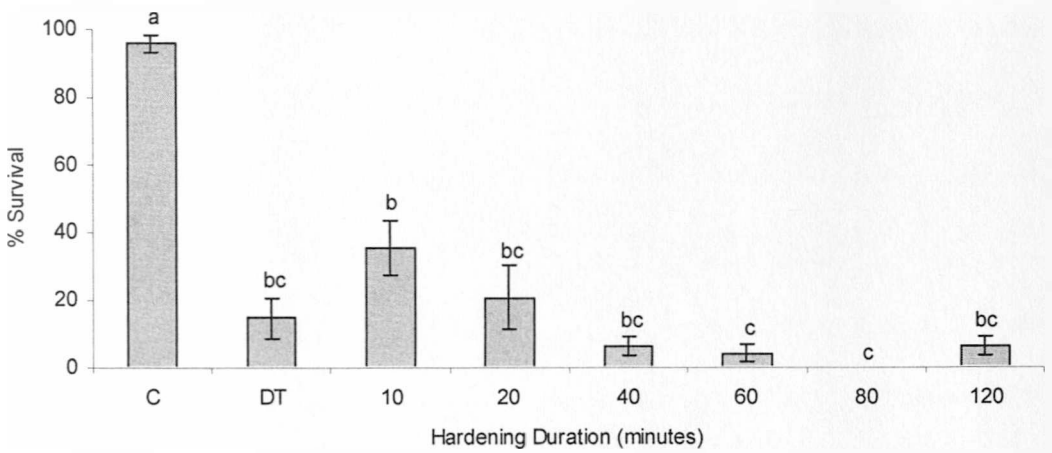

Figure 3.17 Effect of hardening on adult male *Episyrphus balteatus*: survival of -8°C (2h) following hardening at 0°C for 10 to 120 minutes. Error bars show SEM. C = Control. DT = survival following direct transfer to -8°C (2h) without hardening. Means shown with the same letter are not different at p = 0.05.

Adult female. The female showed the same response pattern as the male. The control survival rate was again 98 ± 3%, declining to zero between -4 and -10°C (Figure 3.18). The same discriminating temperature was also used, -8°C, although this yielded a higher mean survival rate in females, 33 ± 10%. Again, 10min hardening increased survival to 52 ± 3.8%, but this was not statistically greater than the direct transfer rate, and survival then declined to below the direct transfer rate as hardening duration increased (Figure 3.19).

3.5 Discussion

E. balteatus does not overwinter in the UK in significant numbers. Migratory adults arrive from the continent during the spring, and others return in the autumn. It is a multivoltine species, with between one and three generations possible in the UK (Hart *et al.*, 1997). If the last generation is to contribute adults to the southward migration, it must complete development during declining autumn temperatures. Active adults are regularly recorded in October and November in the UK (Ball and Morris, 2000), months when 5-10 air frost days are typically encountered (UK Meteorological office data, accessed 2006). The cold hardiness of each life cycle stage in succession may therefore be important, and a relatively cold hardy stage later in the cycle may be compromised by a lack of cold hardiness in an earlier stage, if the two are exposed to the same low temperatures. In the LLT experiments described above, few third instar larvae survived exposure to -10°C, while well over half of pupae survived this temperature. These stages may be only a few days apart in nature, and

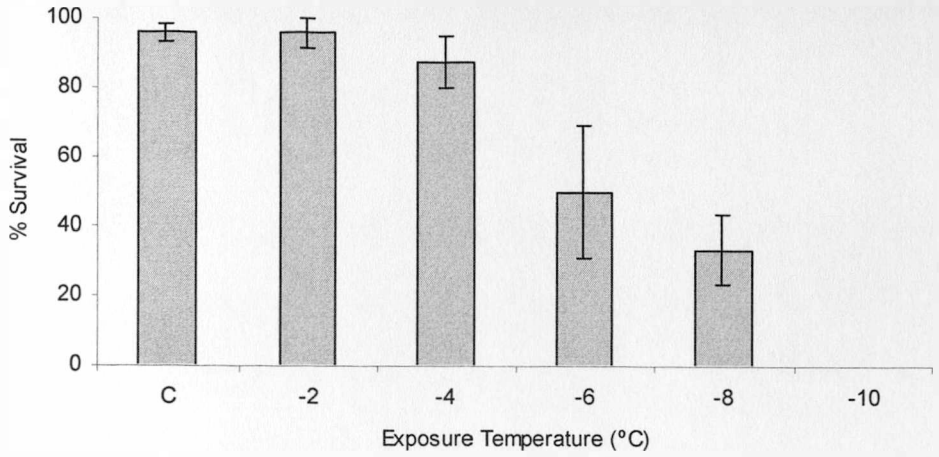

Figure 3.18 Survival of adult female *Episyrphus balteatus* after 2h exposures at -2 to -10°C. C = control. Error bars show SEM.

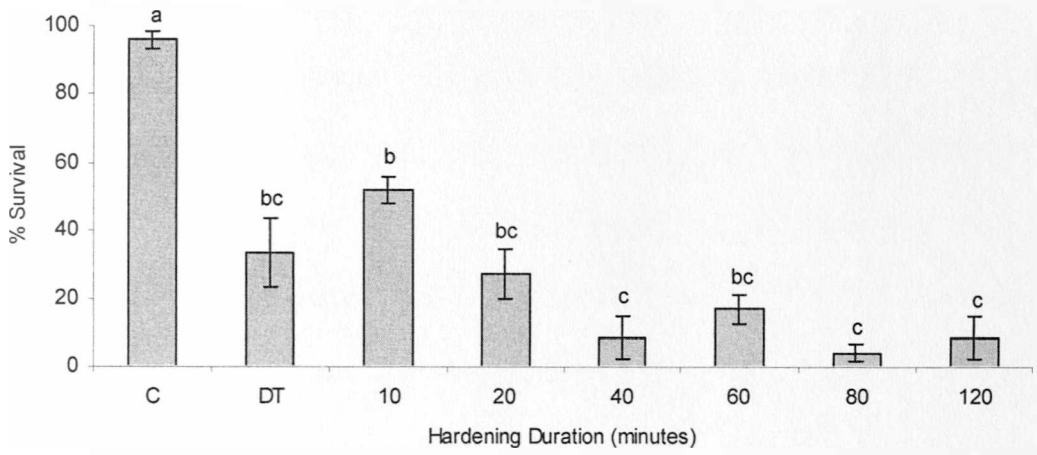

Figure 3.19 Effect of hardening on adult female *Episyrphus balteatus*: survival of -8°C (2h) following hardening at 0°C for 10 to 120 minutes. Error bars show SEM. C = Control. DT = survival following direct transfer to -8°C (2h) without hardening. Means shown with the same letter are not different at p = 0.05.

while -10°C would not normally be experienced in the UK, this nevertheless demonstrates that a weakly cold-tolerant larval instar could act as a survival-limiting stage within the life cycle.

In other species, RCH often takes longer than 10min to develop, and the effect generally increases with increasing hardening durations of the order of 2h. Also, optimal hardening generally occurs at temperatures between 5 and 0°C. These are the effects seen in other Diptera (e.g. *S. crassipalpis* (Chen *et al.*, 1987), *D. melanogaster* (Czajka and Lee, 1990), and *M. domestica* (Coulson and Bale, 1990). However, in *E. balteatus* optimal RCH was induced by very short periods of hardening at 0°C. With the exception of pupae, the maximum response followed just 10min at 0°C, and the effect declined with increasing hardening duration, falling to zero at 80min hardening. In nature, slow cooling rates are likely to be equivalent to the longer hardening durations, and any initial benefit could thus be lost in this weakly cold-tolerant species. However, temperatures around 0°C may rarely be encountered, particularly given the migratory avoidance of low temperatures demonstrated by *E. balteatus*. For this reason, RCH at lethal temperatures may have limited ecological function in *Episyrphus balteatus*, and hardening at higher, behavioural thresholds may be more important. This hypothesis is examined further in Chapters 4 and 5.

Furthermore, in the work presented in this chapter, a single hardening temperature (0°C) was used due to restraints on time and resources, and because the principle goal was the comparison of life stages. However, hardening at temperatures other than 0°C, or by gradual cooling, may induce RCH of different magnitude; this is also examined further in Chapters 4 and 5.

The supercooling point (SCP) profile through successive life stages was characteristic of elevating SCP with increasing levels of ice nucleators ingested in the diet, through to gut evacuation and pupation, where nucleators are egested and/or masked. SCP did not change significantly within approximately 6h either side of the moult from L2 to L3, indicating that SCP in larvae is not dependent on instar *per se*, but rather on age, probably through nucleator build-up. Following on from the pupa, SCP was again elevated in the (feeding) adult and lowest in the egg, where nucleators are masked or absent. This pattern was similar to that shown in other species (e.g. by Coulson and Bale, 1990).

The SCP distributions for each life stage are shown in Figure 3.2. Although the dispersion for some life stages was broad, most notably in the first larval instar, the SCP distributions were unimodal. Bimodality in SCP distributions has been reported in certain polar arthropods, such as the collembolan *Cryptopygus antarcticus* (Block and Worland, 2001), and may relate to variation in the proportions of moulting and non-moulting individuals in the population at any given time (Worland *et al.*, 2006). However, not all species in which SCP distributions are investigated show bimodality (e.g. the beetles *Hydromedion sparsutum* and *Perimylops antarcticus* (Worland *et al.*, 1993)), and in *E. balteatus*, particularly in the actively feeding stages, irregularity in SCP distributions may relate to the stochastic nature of freezing in supercooled liquids (Bigg, 1953), or variations in body mass or nucleator presence (see Lee and Constanzo, 1998).

SCP is known to vary with mass in many invertebrates, and most studies on this subject have measured wild-caught individuals (Lee and Constanzo, 1998; Waller *et al.*, 2006). This study attempted to test the relationship in *E. balteatus* by rearing a group of adults which spanned

close to the maximum possible range of masses in this species, from starved individuals (resulting in minimum viable adult mass) to those fed *ad libitum* during rearing, in the laboratory. In nature, stochastic variation in food supply can lead to considerable variation in mass (and age at maturity) in adult Syrphids (Tenhumberg *et al.*, 2000). There was a significant positive relationship between mass and SCP in adult males, although within the model fitted, only 10% of the variation in SCP was explained by correlation with mass, and the relationship was not found in females. Body mass is therefore unlikely to be a major determinant of SCP in adult *E. balteatus.* However, the same rearing approach could be used to provide a group of larvae in which to model the relationship between mass and SCP. Insufficient numbers of larvae were available to investigate this during the present study, but the greater overall mass of, in particular, third instar larvae, might enable a larger range of masses to be achieved under controlled conditions.

Freezing of the leaf at around -12°C did not cause the eggs laid on it to freeze; this occurred some 13°C lower, indicating that inoculation of freezing from the leaf across the egg membrane does not occur. The significant supercooling capacity of the egg might suggest it as a putative overwintering stage, but in the absence of lethal time data, it is not possible to conclude further.

No individuals survived freezing, and in all stages most individuals appeared to be killed by chilling-injury, at temperatures above the SCP. However, in some life stages, particularly the third instar, the mean SCP lies within the LLT range, indicating that a proportion of the sample mortality is due to freezing. While this may suggest a degree of chill tolerance, under these experimental conditions only brief exposures were used, and lethal time was not

investigated. The relatively high SCPs, and survival down to the SCP under this type of protocol, are in agreement with the results for adults by Hart and Bale (1997b).

In many insects, the SCP is not a reliable indicator of overall cold hardiness, due primarily to the occurrence of chilling-injury at temperatures above the SCP (Bale, 1987; Somme, 1996). The LLT, measured by for example the LT_{50}, gives a better measure of cold hardiness, identifying chilling injury, and incorporating mortality due to freezing if it occurs. However, in this study, comparison of the SCP and LLT within the different life stages of *E. balteatus* showed that the relative positions of the SCP and LLT are not fixed. The range of SCPs was generally broad, with a few individuals freezing at temperatures well below the majority. This variation may to a certain extent reflect the essentially stochastic nature of freezing in supercooled systems. The range of SCPs meant that a number of individuals were killed by freezing within the LLT range, and that the ratio of these to those killed by chilling injury also varied.

Comparing mean SCP to the LLT range (Figure 3.4) provides examples of three scenarios: (1) SCP significantly lower than LLT (egg); (2) SCP at lower extremity of LLT, close to LT_{90} (L1, L2, pupa, and adult female); and (3) SCP centred in LLT range, close to LT_{50} (L2 and adult male). Based on these relationships, it would be expected that, in case (1), most mortality would be due to chilling injury; in case (2), most individuals would be killed by chilling injury, but a few remain alive until freezing; and in case (3), significant mortality through both mechanisms.

In the first and second instar larvae, and the pupa, scenario 2 appeared to apply: the SCP was

close to the LT_{90} and each showed a strong RCH response (Figures 3.8, 3.10 and 3.14). Conversely, in the third instar larva, the SCP lay close to the LT_{50} (scenario 3), and the variable results of the hardening treatments were consistent with a proportion of the sample being killed by freezing (Figure 3.12).

The results for adults were similar in both sexes, as reported in other species which show a strong RCH response (e.g. the olive fruit fly *Bactrocera oleae*; Koveos, 2001). However, in *E. balteatus*, the adults did not show significant hardening. The optimal hardening duration increased survival by slightly more in females (19%) than males (15%), but this was not statistically significant in either case. While this may have been expected in the male, where the SCP lay close the LT_{50}, the converse would have been expected in the female. It is possible, however, that hardening at 0°C is not an effective treatment in the weakly cold-tolerant adults, but rather that this temperature represents a significant stress in itself. Hardening at 10°C has been shown to be significantly more effective at inducing RCH at the flight threshold (see Chapter 4).

Based on the above criteria, the egg has the strongest potential for RCH, without being compromised by freezing. RCH did take place, but was weaker than would be expected given the position of the SCP several degrees below the LLT (case 1). This study is believed to be the first to test for RCH in an insect egg, although short-term acclimation (6h to 10d) was shown to increase survival after 3d at -10°C in eggs of *Locusta migratoria* (Jing and Kang, 2003). Low levels of egg fertility are usual in captive Syrphids, due to problems with mating (Gilbert, pers. comm.). It was not found possible to conclusively identify non-fertilised eggs and exclude them from the samples in this study. In nature, where egg fertility is likely to be

higher, RCH effectiveness may be different to that shown in this study. It is not known to what degree RCH is dependent on the development status of the egg.

The data for pupae (Figure 3.14) suggest a possible bimodal response. This could be effected by a combination of two mechanisms, one induced very rapidly, and one by longer hardening durations. Such a phenomenon is not typically observed in other species in survival experiments, but a two-stage process of this type has been suggested in explanation of the biphasic nature of increasing chill-coma recovery time with decreasing recovery temperature in *D. melanogaster* (David *et al.*, 2003). However, further experiments would be required to determine the effect of longer hardening durations in *E. balteatus* pupae and identify whether or not the bimodal response was an artefact.

The effectiveness of RCH also appears to depend on the temperature to which a sample is exposed. This was demonstrated in pupae of *E. balteatus.* At the -8°C exposure, the optimal hardening duration increased survival by approximately 50%, from 30% to 80% (Figure 3.14). At -7°C however, RCH increased survival by only 24%, from 66% to, again, 80% (Figure 3.15). This suggests that RCH did not operate in all individuals within the sample. It is possible that the remaining 20% in *E. balteatus* pupae were killed by freezing. The relevance of RCH to survival of cold shock injury within samples may be therefore be reduced at less extreme cold shock temperatures.

This analysis suggests that RCH, which acts to prevent chilling-injury but does not affect the SCP (excepting in certain polar species), may be of variable benefit to different individuals and/or life stages. Where the SCP lies below, or at the lower end of the LLT range, there are

more 'available degrees' through which RCH can act to depress the LLT before the SCP is reached. By contrast, in samples where mortality is caused by both chilling-injury and freezing, RCH may by definition be less effective. In species or life stages where the mean SCP lies within the LLT range, it is likely that there will be some temperature overlap between freezing and non-freezing mortality. Only in those individuals with a temperature interval between their LLT and SCP will RCH be possible. However, in such cases, and where the SCP is unaffected by RCH (as is the case in most species), the ability to detect RCH experimentally is restricted, as any depression of the LLT is masked by the freezing event and resulting mortality. The more the SCP range overlaps the LLT range, the less effective RCH can be. The ability of certain polar species (discussed above) to depress the SCP is of clear adaptive significance, lowering this limit. In most species however, freezing temperature is a major constraint on the ability to rapidly cold-harden, and in individuals with high SCPs, such as actively feeding stages, RCH may be effectively overridden.

Chapter 4

Rapid cold-hardening at the flight threshold in *Episyrphus balteatus*

4.1 Introduction

Ambient temperature is a key limiting factor of flight activity in a wide range of insect species, and is a prime determinant of the daily activity of dipterans in nature (Peng *et. al.*, 1992). Flight temperature thresholds have been determined for many insects, both in the laboratory and by correlating air temperature with observed activity in the natural environment, but the influence of cold-hardening on this threshold is not well understood.

Examples of temperature-dependent flight activities in nature include the dispersal of stoneflies between different stream populations (Briers *et al.*, 2003), daily movement of damson-hop aphid *Phorodon humuli* to and from commercial hop gardens (Campbell and Muir, 2005), and the formation of mating swarms in the dance fly *Empis borealis* (Svensson and Petersson, 1995). Migratory insects in the atmosphere are often located at the altitude of the warmest air. This has been shown by radar observation of moths over the UK (Reynolds *et al.*, 2005; Wood *et al.*, 2006), although similar observations over China indicated wind speed and sheer as more important than air temperature (Feng *et al.*, 2005).

In addition to temperature, a number of other environmental factors affect insect flight. Many species have been shown to decrease flight at low ambient light levels, e.g. hornets (Spiewok and Schmolz, 2006). Low air pressures, such as those encountered in high

altitude alpine environments, decreased flight proclivity in *Drosophila melanogaster* (Dillon and Frazier, 2006). This effect was also more pronounced at low temperatures. Humidity may also have a role. For example, in the beetle *Euophryum confine*, low humidity increased flight at optimal temperatures, although at high temperatures low humidity increased mortality (Green and Pitman, 2003). Daily abundances of dipterans in suction traps was dependent on temperature in day-flying species, but associated with wind speed in night-flying species (Peng *et. al.*, 1992). The degree of 'need' for flight may also play a role. For example, starvation increased flight activity in the beetle *Protostephanus truncatus* (Fadamiro and Wyatt, 1995).

In some cases, different environmental factors are involved in commencement, maintenance and cessation of flight during daily foraging cycles. For example, in the dwarf honeybee *Apis florea*, flight activity commenced when a temperature threshold had been met, and the numbers of individuals foraging was positively correlated with temperature during the day, but cessation of foraging in the evening was mainly associated with decreasing light intensity, not temperature (Abrol, 2006). As well as the initiation of flight, flight speed has been shown to positively correlate with air temperature in some species, such as the wax moth *Galleria mellonella* (Schmolz *et al.*, 1999).

The mechanisms by which temperature affects insect flight are complex. Air temperature strongly affects the metabolic rate during flight in some species (Harrison and Roberts, 2000). One of the primary modes of action is the temperature-dependent performance of the flight muscles. For example, the power output of bumblebee flight muscle is strongly temperature-dependent (Gilmour and Ellington, 1993). This may be related to enzyme

function. The catalytic efficiency of hexokinase, involved in energy supply to the wing muscles of *Dipetalogaster maximus*, increases with temperature to a maximum at around 37°C (Scaraffia and De Burgos, 2000). Neural function can also be affected by temperature. For example, synaptic transmission in flight muscle circuits of *Locusta migratoria* is temperature sensitive, and can be protected by heat shock against damage at high temperatures (Dawson-Scully and Robertson, 2000). Heat shock proteins, including HSP70, are likely to be involved (Robertson, 2004).

While ambient temperature limits flight in many species, an insect's body temperature is not necessarily in equilibrium with that of the surrounding air. Basking (to absorb solar radiation) can elevate body temperature above ambient. The bombyliid fly *Usia aurata* employs basking, and also absorbs heat from the heliotropic flowers on which it basks, which themselves elevate their surface temperatures by absorption of solar radiation (to the benefit of pollen germination). The fly spent significantly more time feeding and less time basking on flower heads whose own surface temperature was higher (Orueta, 2002). Various species elevate their body temperature by beating the wing muscles before initiating flight. One species of cicada, *Tibicen winnemanna*, beats its wing muscles in this manner (an 'endothermic' behaviour), and is thus less dependent on high ambient temperatures for initiating flight than the related 'ectothermic' species *T. chloromerus*, which uses basking to increase body temperature (Sanborn, 2000).

During flight, body temperature is determined by the rates of heat generation (by metabolic activity) and loss (through convection and evaporative cooling) (Gilbert, 1984). These are further influenced by air temperature, solar radiation and wind speed (Bishop and Armbruster, 1999). Many insects, while ectothermic at rest, become endothermic

during flight; heat generated by the flight muscles raises the body temperature above that of the surrounding air, and the effect is generally greater in insects of larger body size. Thermoregulation during flight is particularly well characterised for the bees and wasps. For example, orchid bees, such as *Euglossa imperialis*, regulate heat production and hence body temperature by increasing wing-beat frequency in response to declining ambient temperature (Borrell and Medeiros, 2004). However, wing-beat frequency in some dipterans shows a linear dependency on ambient temperature (Azevedo *et al.*, 1998). Heat production during flight in some species is so great that a degree of active cooling may be required, in order to maintain optimal body temperature for operation of the flight muscles. This may be the case in some Odonata, where changes in haemolymph circulation in the thorax may improve heat loss (May, 1995).

Most hoverflies are not endothermic in flight, due largely to their small body size, but utilise basking and beating of the wing muscles before flight to raise the body temperature (Gilbert, 1984). However, evaporative cooling mechanisms can outweigh heat production and lower body temperature below ambient. This occurs in hoverflies, including those of the *Eristalis* genus (Bressin and Willmer, 2000), and *Syrphus ribesii* (Gilbert, 1984). The black and yellow colour pattern of adult *Episyrphus balteatus* is known to be very plastic, and lower temperatures during the pupal stage result in darker adults (Marriott and Holloway, 1998). It has been hypothesised that this may have a thermoregulatory role in cooler environments by increasing the absorption of solar radiation (Gilbert, 1984), but this has not been demonstrated experimentally in hoverflies. It has, however, been shown that darker phenotypes within other species warm up faster under laboratory conditions (e.g. speckled wood butterfly *Pararge aegeria*; Van Dyk and Matthysen, 1998).

Some studies have identified sex differences in the effect of temperature on locomotion. Flight speed in drones (males) of the hornet *Vespa cabrio* was dependent on ambient air temperature, but that of workers was not (Spiewok and Schmolz, 2006). Males of two species of eucalyptus-boring beetles (genus *Phorocantha*) had significantly higher metabolic rates during running than did females, consistent with the higher activity level of males during mate-seeking behaviour in these species (Rogowitz and Chappell, 2000).

In some cases, habitat temperature may have applied adaptive pressure to the mechanics of flight. Wing size (relative to thorax size) increased significantly with latitude in wild-collected *Drosophila melanogaster*, suggesting that increased wing-size may be selectively advantageous at low temperatures (Azevedo *et al.*, 1998). The converse is the case in high altitude alpine environments where it has been suggested that the limits placed on flight by low temperatures may have contributed to the prevalence of aptery in alpine species (Dillon and Frazier, 2006).

The relationship between air temperature and insect flight has been applied to agriculture in various studies, such as adjusting the temperature of livestock housing for the control of dipteran pests (Lysyk, 1995), predicting flight activity of the rusty grain beetle *Cryptolestes ferrugineus* at commercial grain stores (Nansen *et al.*, 2004), and identifying bee species of potential use as pollinators (Corbet *et al.*, 1993).

While the effect of cold-hardening on survival at the lower lethal limit has been well demonstrated, its role at other low temperature thresholds has not been well characterised. Rapid cold-hardening (RCH) in insects is typically characterised as a process whereby a brief period of acclimation at temperatures around zero allows increased survival of

subsequent exposure to otherwise lethal sub-zero temperatures (Lee *et al.*, 1987). This process has been demonstrated in a range of species. However, a few studies have shown that RCH operates at temperature thresholds other than the lower lethal limit, particularly the temperature at which insects enter torpor (Meats, 1973; Powell and Bale, in press). Meats (1973) described a continual, rapid process of "reacclimatization" of torpor temperature during cooling at rates up to 1°C min^{-1} in the fruit fly *Dacus tryoni*. Powell and Bale (in press) found that RCH decreased mean torpor temperature by up to 2.8°C in the grain aphid *Sitobion avenae*. The effect of RCH on the time taken to recover from chill coma has also been investigated in *Drosophila melanogaster* (Rako and Hoffman, 2005), where no effect was found.

While there have so far been few investigations of the effect of RCH on thresholds other than the lethal limit, the above shows that this new area of enquiry may provide useful insight into the functional mechanics of the RCH process, as well as its relevance to maintaining locomotion at low temperatures. The following study examines the effect of RCH on the flight threshold of the hoverfly *Episyrphus balteatus*. This species does not overwinter in the UK in significant numbers, but migrates from southern Europe in the Spring (Ball and Morris, 2000). It is multivoltine, with between 1 and 3 generations possible in the UK, and adults of the last generation are thought to return south in some numbers in the Autumn; large movements have been recorded by trapping at a number of locations across Europe (Owen, 1956; Sutton, 1969; Aubert *et al.*, 1976; Aubert and Goeldlin, 1981; Gatter and Schmidt, 1990). Given the known influence of temperature on insect flight and migration, the flight threshold in this species is likely to have a role in determining its phenology in the UK, particularly its dispersal behaviour on arrival in the Spring, and migration in the Autumn. *E. balteatus* is known to be only weakly cold-

tolerant, and it is reasonable to conclude that it migrates to avoid low winter temperatures across much of its range (Hart and Bale, 1997b). For this reason, it is the species' low-temperature flight threshold, rather than its lower lethal temperature, that is likely to be most relevant to successful migration in the Autumn. It can also be hypothesised that the ability to rapidly cold-harden at the flight threshold would be of benefit in meeting unpredictable variations in temperature encountered during migration over long distances.

4.2 Aims

- Determine upper and lower temperature thresholds for flight in *E. balteatus*, in the laboratory.
- Assess the effect of various cold hardening treatments on flight at sub-optimal temperatures, and test the hypothesis that RCH will increase flight at low temperatures.
- Calculate 'FT_{50}' values, the temperature at which 50% of a sample flies within a given time, equivalent in concept to LT50 (lethal temperature) or ED50 (effective dose) values used in other areas of research. Use of such 'Flight Temperature 50' values to quantify the threshold temperature for flight may allow meaningful testing of the hypothesis that RCH 'lowers' that threshold.
- Differentiate between the responses of the two sexes.

4.3 Methods

Eggs were collected from the laboratory culture in 24h batches, and the resulting larvae reared on *Aphis fabae*, at 20°C and 18L:6D photoperiod. From each batch, new adults emerged over 2-5 day periods. Newly emerged adult flies were collected at daily intervals and provided with water, sugar and pollen while awaiting use in experiments, which took place 24-34 hours after eclosion.

4.3.1 Determining flight proclivity

The number of individuals flying at a given air temperature was assessed after the method of Gould (1998). A straight-sided glass dish (8cm diameter x 4cm deep) was prepared by coating the vertical inner surfaces with talcum powder. Flies placed in this dish were unable to walk out over the powder, but could readily fly out. The outside of the dish was also blacked out with dark card.

Samples of flies were placed in glass boiling tubes and subdued by chilling in ice water for 4 minutes – the minimum time necessary for all to enter chill coma. The flies were then placed in the glass dish and inserted into a 30x30x30cm muslin cage, itself inside an incubator maintaining the desired air temperature. Food and water was provided, and the interior was well lit. After 2h, the number of flies that had flown out of the dish was recorded. In treated groups, hardening treatments were first applied in a refrigerated bath. Those exposed at (or by cooling to) 10°C were then placed in ice water for 4 minutes to induce coma before placing them in the dish and incubator as before. The ice-water stage was omitted for those samples treated at (or by cooling to) 0°C, as these flies were already in coma. Sample size was 10, with 5 replicates per temperature per treatment. Males and

females were treated independently.

4.3.2 Hardening Treatments

Two types of hardening treatments were used: direct transfer (where flies were moved directly from rearing temperature to hardening temperature), and gradual cooling (where flies were cooled at slower rates). Direct transfer hardening was carried out at either 0°C or 10°C, and for varying durations (10, 30, 60 or 120 minutes). Hardening by gradual cooling was carried out by cooling from 20°C to either 0°C or 10°C, at varying rates (1, 0.5, 0.1 or 0.05°C min^{-1}).

In order to more clearly ascertain the effects of hardening at 10°C (by direct transfer or gradual cooling), these experiments were repeated, omitting the ice water stage of the protocol. While this stage is necessary to facilitate the placement of non-hardened flies into the dish and incubator, 4 minutes at approx. 0°C cannot be discounted as a treatment in itself. Although not in coma following treatment at 10°C, flies were sufficiently subdued to enable placement into the incubator. The ice water stage was therefore omitted for these experiments to create a treatment protocol more closely approximating temperature changes in nature, and to give an indication of whether or not inclusion of the ice-water stage in the experiments with non-hardened flies caused a bias in those results. FT_{50} values were then calculated for two treatment groups as in phase one, again omitting the ice water stage. The hardening treatments applied in this study are summarised in Table 4.1.

Table 4.1 Organisation of hardening treatments used in flight threshold experiments conducted with adult *Episyrphus balteatus*.

	Direct Transfer	Gradual Cooling
0°C	10, 30, 60 & 120min at 0°C	Cooling from 20 to 0°C at 1, 0.5, 0.1 & 0.05°C/min
10°C	10, 30, 60 & 120min at 10°C	Cooling from 20 to 10°C at 1, 0.5, 0.1 & 0.05°C/min
10°C ice-water stage omitted	10, 30, 60 & 120min at 10°C	Cooling from 20 to 10°C at 1, 0.5, 0.1 & 0.05°C/min

Three sets of experiments were carried out:

(1) Flight in non-hardened flies was recorded at temperatures from 10 to 40°C, in 2.5°C intervals. After exposure at a selection of high air temperatures, flies were placed in small recovery cages to record survival over 72h; a handling control was carried out for this (survival > 95%).

(2) A single air temperature yielding approximately 20% flight was selected (the so-called 'discriminating temperature') at which to assess the effect of the hardening treatments and test for RCH.

(3) Based on the results of (2) above, four treatments which yielded a significant increase in flight were selected and their effect examined across a range of sub-optimal air temperatures. This data was then used to calculate an 'FT_{50}' value, defined as the air temperature for 50% of the sample to fly.

At no stage were any individual flies included in experiments more than once.

4.3.3 Statistical analysis

Percentage flight data was arcsin transformed and the results compared with two-way ANOVA and Tukey's multiple comparisons preserving family error rate, within the general linear model procedure of the statistical software package Minitab (Minitab Inc., 2003). This procedure allowed sex-differences and treatment-differences to be clearly identified, independently of each other (i.e. with the other effectively 'pooled') for simplified comparison with other studies that do not treat the sexes independently; these analyses are presented as significance letters on the graphs that follow. When there was no significant sex*treatment interaction (all cases), sexes were then treated separately with one-way ANOVA and Dunnett's test to test each treatment against its control; these are shown as asterisks on the graphs that follow. RCH was thus identified by a significant increase in flight over the control value.

Negative binomial regression was used to calculate FT_{50} values. While other procedures, especially probit analysis, are more commonly used to calculate 50^{th} percentile values from dose-response curves, such as those obtained in response to cold at the lethal threshold, the data obtained in this study's flight threshold experiments did not fit the sigmoidal relationship assumed by probit analysis (i.e. probit transformation did not sufficiently linearise the data to allow regression). Analysis was attempted with a number of other distributions, but no model was significantly fitted. In such circumstances, negative binomial regression, which assumes no such distribution, is an appropriate procedure (Berkvens, pers. comm.).

4.4 Results

4.4.1 Flight in non-hardened flies

In non-hardened flies, the optimal air temperature for flight was in the region of 30°C, and the minimum was 10-12.5°C (see Figure 4.1). Above 27.5°C, flight during the experiment remained high, but survival of the exposure declined with increasing air temperature, with no survivors after exposure to 40°C. This is likely to be related in part to experimental design: beginning the exposure in coma, an individual's body temperature must have warmed through the zone optimal for flight before reaching the lethal air temperature. Although survival was monitored over 72h, there was in fact no delayed mortality, i.e. those individuals that survived the 2h exposure all remained alive three days later. This indicates that the functional upper threshold for flight is 30-32.5°C.

The sexes showed the same percentage flight and declining survival at optimal and above-optimal air temperatures. However, at lower air temperatures there was a marked difference: below 22.5°C, more females flew than males. This difference was greatest between 15 and 22.5°C.

4.4.2 Effects of hardening treatments 1: ice-water stage included

Based on the above results, an air temperature of 15°C was selected as the discriminating temperature at which to test the effects of the hardening treatments. In non-hardened flies, 24% of males and 38% of females flew during 2h at 15°C. These data were used as a common control for the hardening treatments.

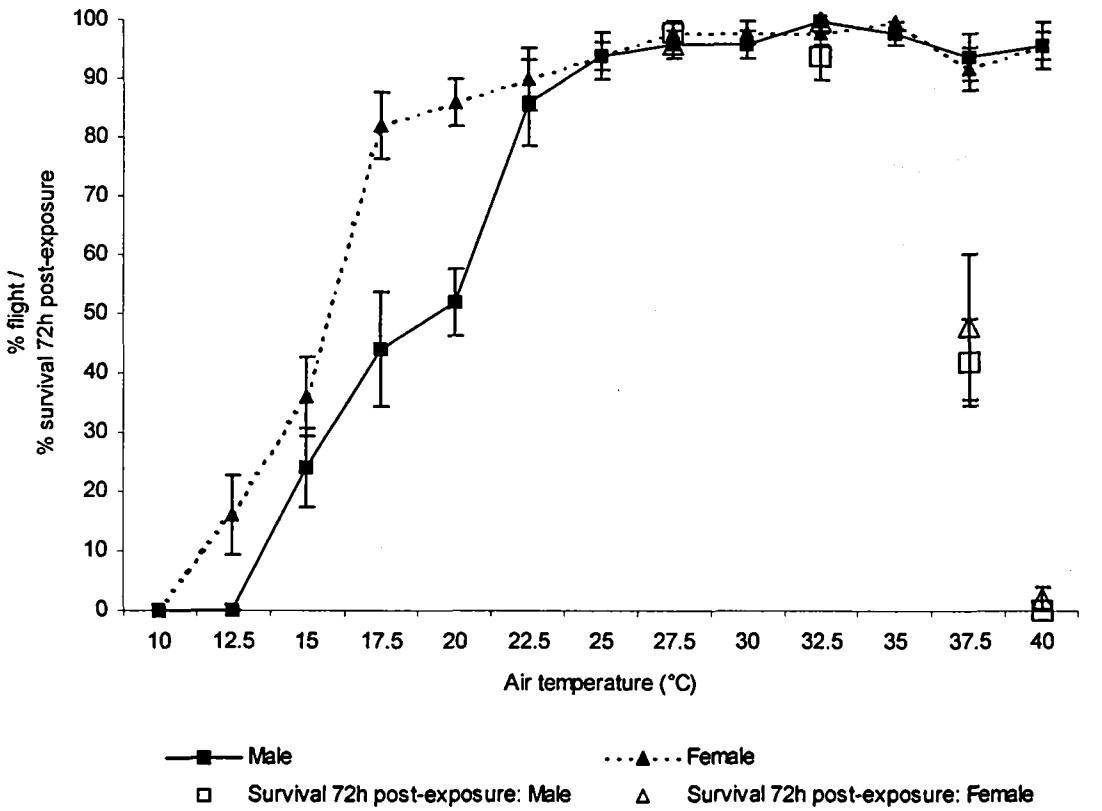

Figure 4.1 The relationship between air temperature and flight threshold in non-hardened adult *Episyrphus balteatus*: mean percentage of the sample that flew during 2h at each temperature. Error bars show SEM. While considered as 'non-hardened', these flies were placed in ice-water for 4 min to induce chill coma (see methods, main text).

The effect of direct transfer hardening at 0°C for various durations is shown in Figure 4.2. The overall difference between sexes was significant ($p<0.01$), as was the treatment effect ($p<0.01$), and there was no sex*treatment interaction ($p=0.412$). However, the effect of treatment was not as expected. In females, no duration significantly increased flight over the control, but the shape of the response was typical of RCH, i.e. the response (flight) increased with increasing hardening duration, to a maximum at an optimal duration (here 30min), above which the response declined again to the control level. In males, however, flight declined in a linear fashion with increasing hardening duration, becoming significantly lower than the control at the 120min treatment.

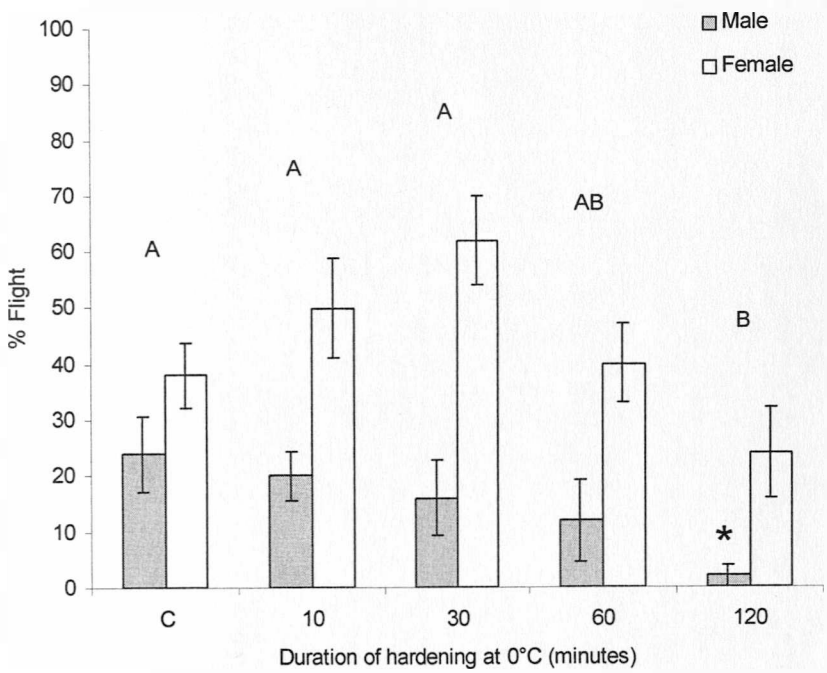

Figure 4.2 Flight at 15°C in *Episyrphus balteatus*: effect of direct transfer hardening at 0°C. Error bars show SEM. C = control (non-hardened flies). Treatments shown with the same letter are not different at $p=0.05$ (independent of sex). Means indicated by an asterisk are different ($p<0.05$) from their corresponding (male or female) control.

By contrast, the results of hardening at 10°C indicated a clear RCH response (Figure 4.3). The response of the sexes were significantly different ($p<0.01$), and there was no sex*treatment interaction ($p=0.295$). Independent of sex, the 60 minute treatment significantly increased flight over the control. Both sexes showed the typical shape of response (as described in the previous paragraph), but the overall effect was greater in females. In males, no treatment resulted in a statistically significant increase in flight, but in females 30 and 60 minutes at 10°C significantly increased flight (from 38%) to 74 and 80% respectively, indicating rapid hardening.

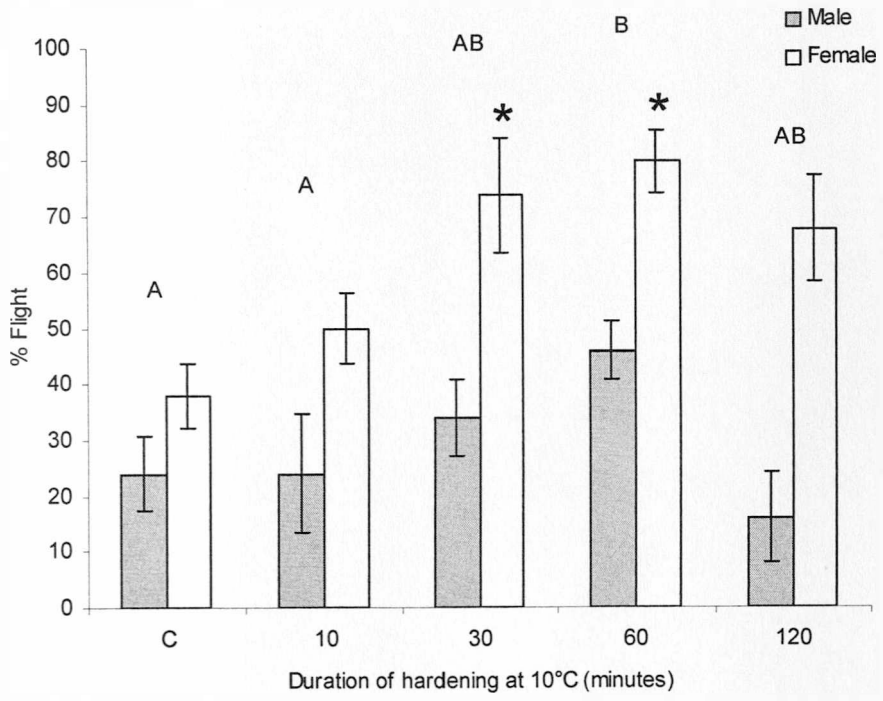

Figure 4.3 Flight at 15°C in *Episyrphus balteatus*: effect of direct transfer hardening at 10°C. Error bars show SEM. C =control (non-hardened flies). Treatments shown with the same letter are not different at $p=0.05$ (independent of sex). Means indicated by an asterisk are different ($p<0.05$) from their corresponding (male or female) control.

The gradual cooling treatments also induced hardening, whether cooling from 20 to 0°C, or 20 to 10°C (Figure 4.4). In both cases, there was no significant sex*treatment interaction ($p=0.07$ and 0.39 respectively), and, independent of sex, the 0.5°C min^{-1} cooling rate significantly increased flight. Again, overall female flight was significantly higher than male in both cases ($p<0.01$). Within sexes, cooling from 20 to 0°C, flight was significantly increased by the 1.0°C min^{-1} rate in females and the 0.5°C min^{-1} rate in males. When cooling to 10°C, no treatment significantly increased flight in males, but in females the 1.0 and 0.5°C min^{-1} rates resulted in greatly increased flight (Figure 4.5). The slower cooling rates (0.1 and 0.05°C min^{-1}) did not induce significant hardening at any point; indeed, the shape of the response suggests that increasingly slow cooling rates may actually decrease overall flight.

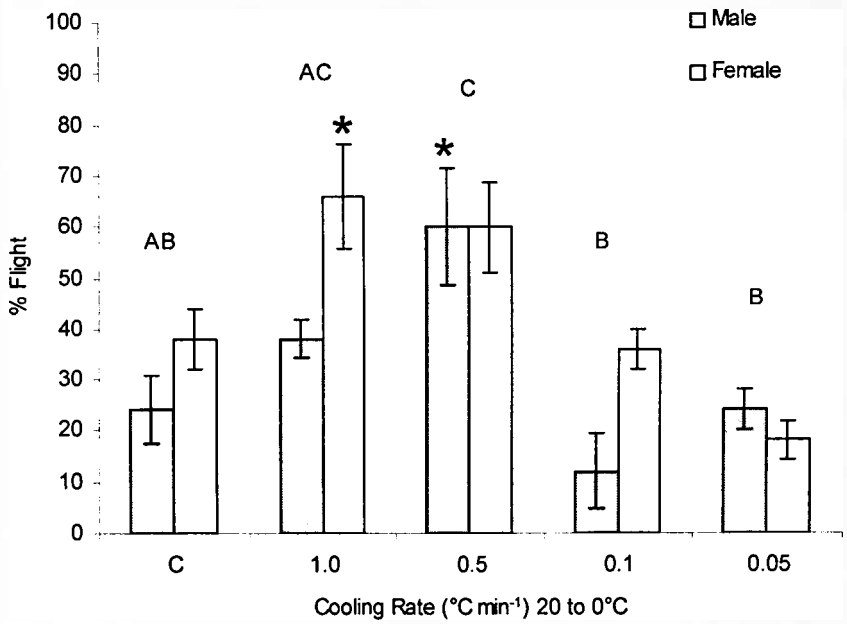

Figure 4.4. Flight at 15°C in *Episyrphus balteatus*: effect of hardening by gradual cooling from 20 to 0°C. Error bars show SEM. C =control (non-hardened flies). Treatments shown with the same letter are not different at $p=0.05$ (independent of sex). Means indicated by an asterisk are different ($p<0.05$) from their corresponding (male or female) control.

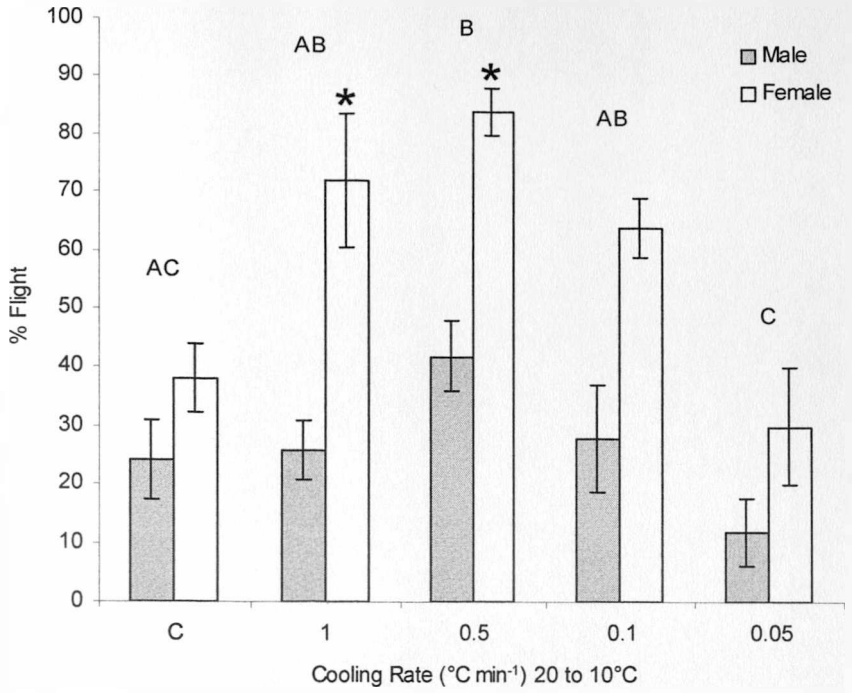

Figure 4.5 Flight at 15°C in *Episyrphus balteatus*: effect of hardening by gradual cooling from 20 to 10°C. Error bars show SEM. C =control (non-hardened flies). Treatments shown with the same letter are not different at p=0.05 (independent of sex). Means indicated by an asterisk are different ($p<0.05$) from their corresponding (male or female) control.

4.4.3 Effects of hardening treatments 2: ice-water stage omitted

The effect of omitting the ice-water stage of the protocol is shown in Figure 4.6. In only two cases did omission of the ice-water stage significantly change mean flight in hardened flies, and in each case flight increased without the ice-water stage. This suggests that the additional 4 minutes in ice-water after hardening treatments at, or cooling to, 10°C did not significantly affect the hardening response, and that the estimates of increased flight in flies hardened by these treatments, and in non-hardened flies, were generally realistic or, indeed, a conservative estimate of the true effect of hardening at 10°C on flight at low temperatures.

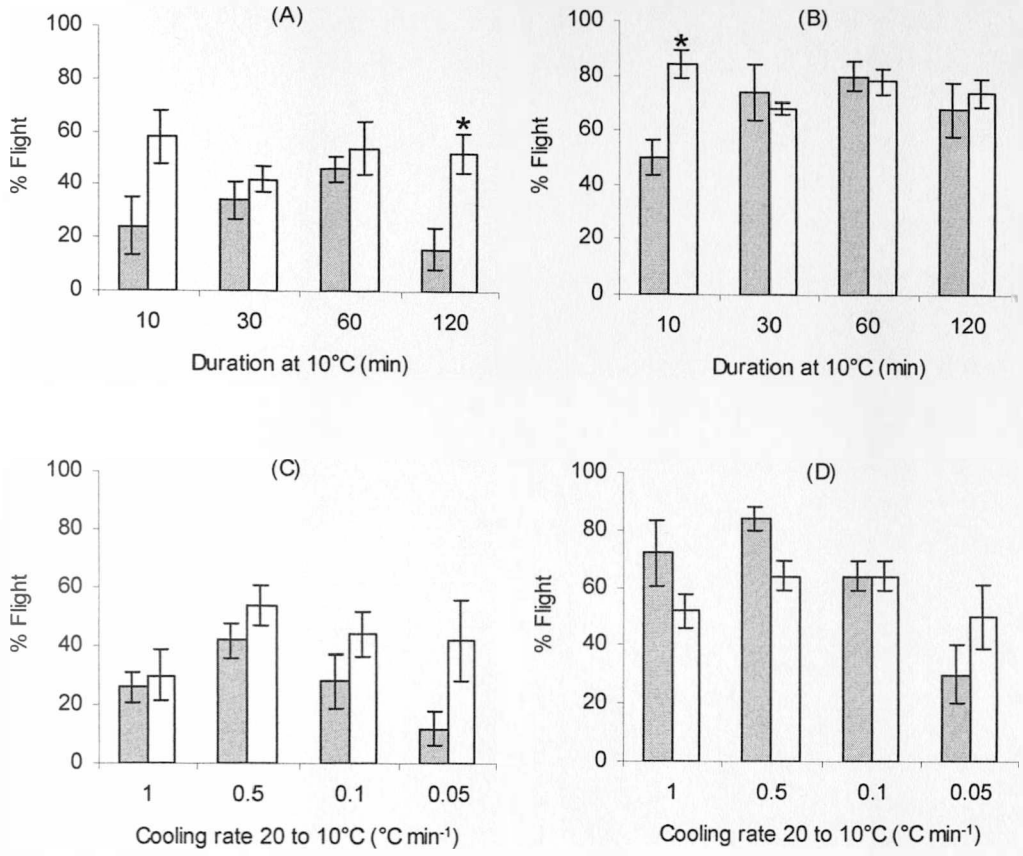

Figure 4.6 Flight in hardened *Episyrphus balteatus*, comparing treatments with the ice-water stage of the protocol included (grey bars) and omitted (white bars). (A) Males, direct transfer hardening at 10°C; (B) Females, direct transfer hardening at 10°C; (C) Males, hardened by gradual cooling from 20 to 10°C; (D) Females, hardened by gradual cooling from 20 to 10°C. Error bars show SEM. Means for ice-stage-omitted treatments indicated by an asterisk are significantly different from the corresponding ice-stage-included mean.

4.4.4 Effects of hardening on the flight threshold: calculation of FT_{50}

Based on the above results, four hardening treatments were selected to test the effect of hardening across the range of sub-optimal air temperatures. First, two treatments including the ice-water stage of the protocol were applied: (1) direct transfer to 10°C for 60 minutes, and (2) cooling from 20 to 10°C at 0.5°C min^{-1}. Next, two 10°C treatments that omitted the ice-water stage were applied: (1) direct transfer to 10°C for 10 minutes,

and (2) cooling from 20 to 10°C at 0.1°C min^{-1}. These were the four *treatments* (independent of sex) that, within their group (direct transfer or gradual cooling; ice-water stage included or omitted), elicited the largest statistically-significant increase in flight over the control. Their effect on flight, compared to the original data for non-hardened flies, is shown in Figures 4.7 to 4.10. There was little difference in effect of the four treatments. In all cases, hardening resulted in a general increase in flight across the threshold temperature range, and the trend of greater female flight than male flight continued.

The FT_{50}'s for each treatment and non-hardened flies are presented in Table 4.2. In non-hardened males the FT_{50} (19.3°C) was significantly higher ($p<0.01$) than that for females (17.5°C). Within each sex, there was no difference between treatment group FT_{50}'s, and the effect of treatment on reducing FT_{50} was the same for both males and females: on average, treatment reduced male FT_{50} by 2.5°C and female FT_{50} by 2.3°C. This reduction was statistically significant in all cases ($p<0.05$).

Table 4.2 Low temperature FT_{50} of male and female *Episyrphus balteatus*, for non-hardened flies and those hardened by each of the four treatments selected. Values followed by the same letter are not significantly different at p=0.05

Treatment	Male FT_{50} (°C)	Female FT_{50} (°C)
Non-hardened	19.3 a	17.5 c
10°C (60min)	16.7 b	14.9 d
Cooling 20 to10°C at 0.5°C min^{-1}	16.9 b	15.1 d
10°C (10min) ice-water omitted	16.7 b	14.9 d
Cooling 20 to 10°C at 0.1°C min^{-1} ice-water omitted	17.1 b	15.2 d

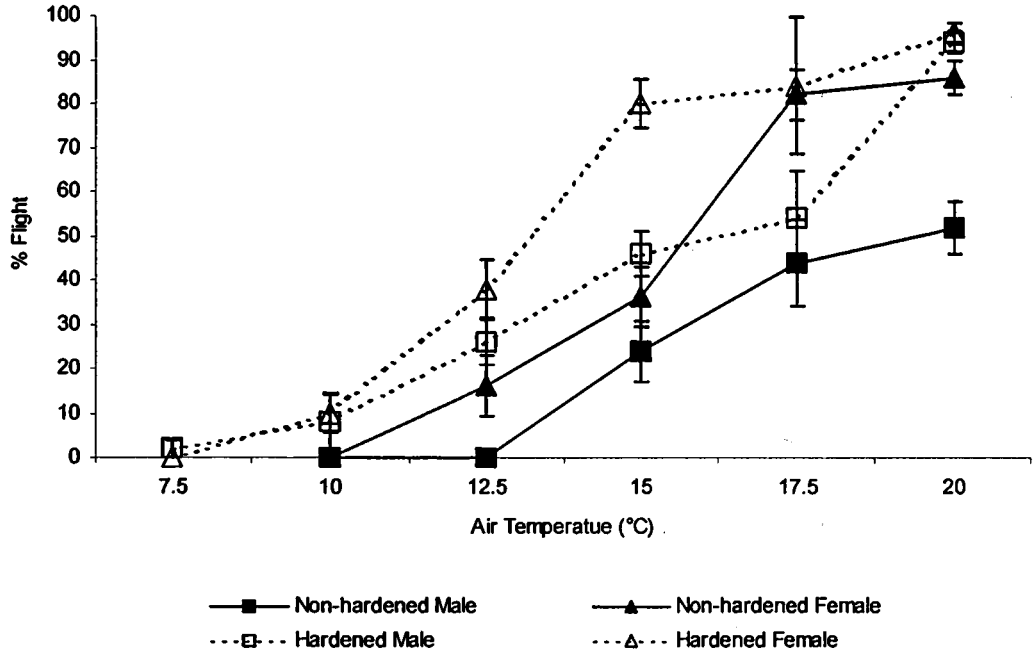

Figure 4.7 Shift in low-temperature flight threshold in *Episyrphus balteatus*: effect of hardening at 10°C (60min). Error bars show SEM.

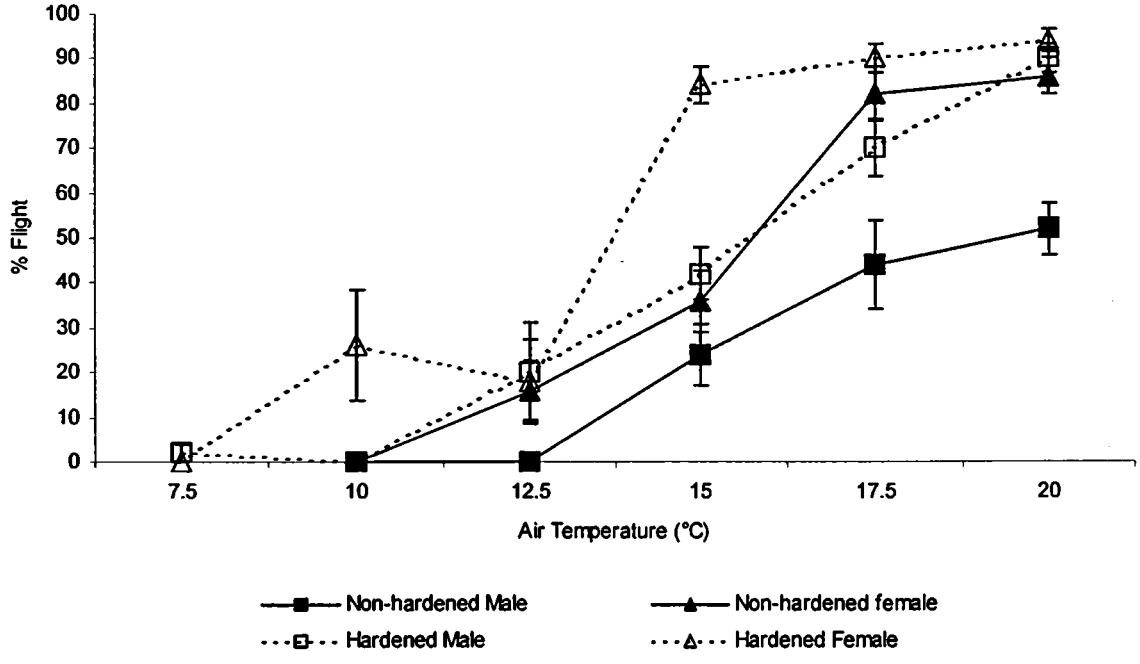

Figure 4.8 Shift in low-temperature flight threshold *in Episyrphus balteatus*: effect of hardening by cooling from 20 to 10°C at 0.05°C min^{-1}. Error bars show SEM.

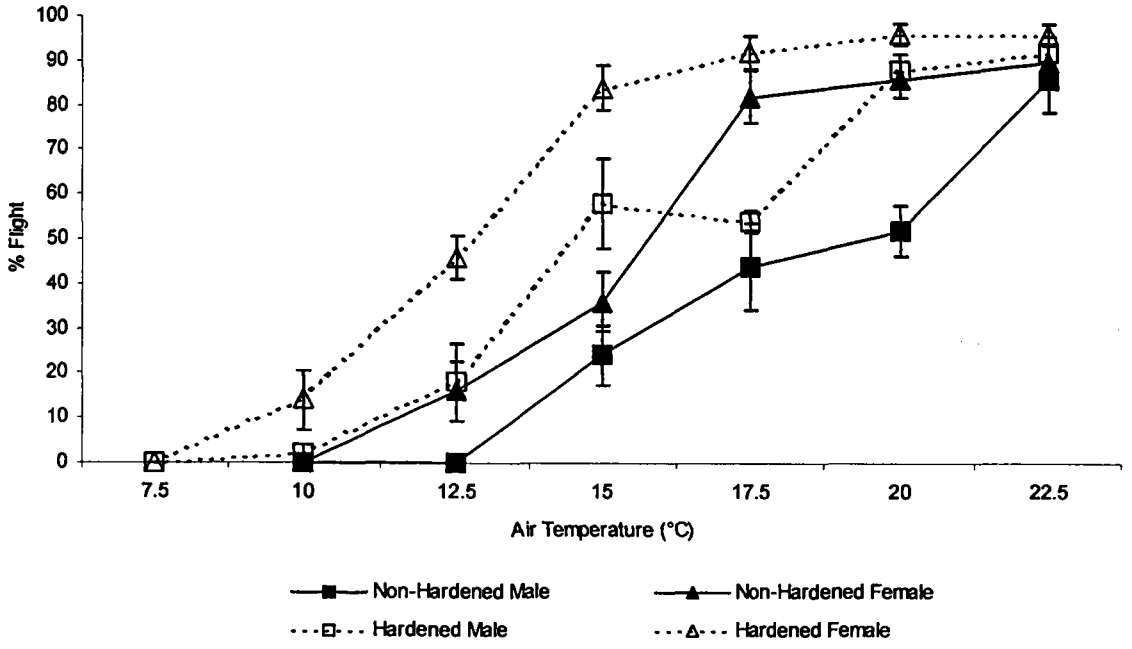

Figure 4.9 Shift in low-temperature flight threshold in *Episyrphus balteatus*: effect of hardening at 10°C (10min), omitting the ice-water stage of the protocol (see methods section). Error bars show SEM.

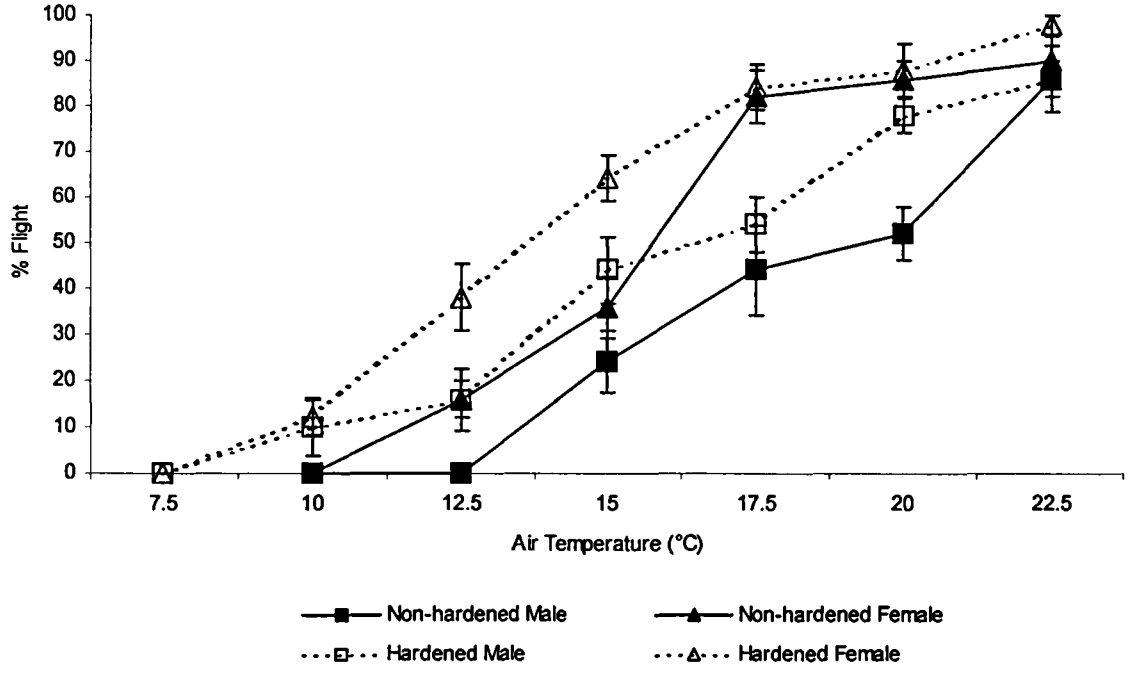

Figure 4.10 Shift in low-temperature flight threshold in *Episyrphus balteatus*: effect of hardening by cooling from 20 to 10°C at 0.1°C min^{-1}, omitting the ice-water stage of the protocol (see methods section). Error bars show SEM.

4.5 Discussion

This is believed to be the first study to investigate the effect of RCH on the flight threshold of an insect, and one in which annual, long-distance migrations are a major feature of the species' ecology. Given its general lack of cold hardiness, and migratory avoidance of low temperatures, the ability of *E. balteatus* to undergo hardening in relation to flight threshold may be of considerable adaptive significance in determining the conditions under which flight is possible, with impacts on the length of the summer season in which it is able to remain in the northerly parts of its range, where overwintering is not possible.

In non-hardened *E. balteatus*, both the low-temperature threshold (10-12.5°C) and optimal temperature for flight (25-30°C) were higher than expected. For comparison, the mean Central England Temperature (an standard index used by the UK Meteorological Office) for June to August for the period 1991 to 2001 was 15.9°C (UK Met. Office data, 2006). It should also be noted that this experimental method determines the initiation of flight under simplified circumstances, rather than capacity for flight *per se*. Preliminary experiments for this study observed that adult *E. balteatus* are able to fly for several metres at 8°C when dropped in mid-air. It is also possible that the well-fed individuals used in this study were less likely to initiate foraging flight than in nature. However, in some species of hoverfly (genus *Eristalis)*, the body temperature is up to 2°C lower than the ambient air temperature, due to evaporative cooling (Bressin and Wilmer, 2000). *E. balteatus* is known to elevate its body temperature by beating the wing muscles before flight, and by basking (Gilbert, 1984). While collecting *E. balteatus* at field sites to establish the laboratory culture used for this study, there was a notable reduction in

activity at flowers when a cloud covered the sun, indicating the importance of solar radiation to activity in this species. Also, 10°C was the minimum temperature at which Gilbert (1985) observed *E. balteatus* during census walks at two garden sites in Cambridge. This evidence suggests that the 7.5 to 10°C lower threshold identified by this laboratory study is realistic.

The results of the hardening treatments support the conclusion that RCH can lower the flight threshold in *E. balteatus*. This is most evident in Figures 4.7 to 4.10; comparing the hardened groups (broken lines) to non-hardened flies (solid lines), all four hardening treatments resulted in a general shift of the relationship upwards and to the left, indicating increased flight at a given temperature, and a decrease in the flight threshold. However, the effects of different hardening regimes were variable, and differed in two principal ways from those found in most studies of RCH.

Firstly, hardening by direct transfer was more effective at 10°C than at 0°C. This was especially evident in males, where flight was in fact significantly negatively affected by hardening at 0°C (Figure 4.2). In males at least, the period at 0°C represented a significant stress. Omission of the ice-water stage of the protocol, thus removing any exposure to 0°C, did further increase hardening to some degree. By contrast, most studies of RCH at the lethal threshold have found that the zone of temperatures in which hardening takes place is around 0°C. This zone may be higher for the flight threshold of *E. balteatus* on account of the threshold itself naturally lying much higher; i.e. the cue temperature for induction of RCH may be determined by the threshold temperature, and may not be the same at lethal and behavioural thresholds.

Secondly, of the cooling rate treatments employed, it was the faster rates (1.0 and 0.5°C min $^{-1}$) that had the greatest effect. This was the case whether cooling to 0 or 10°C, although omission of the ice-water stage from the 10°C treatments did slightly (but not significantly) increase flight after cooling at the slower rates. Queries over the relevance of fast cooling rates, which may not generally occur in nature, have been raised in the literature, and several studies have demonstrated greater hardening effects by cooling at 'ecologically relevant' rates. For example, Kelty and Lee (1999) found significantly higher survival of lethal exposures and significantly lower torpor temperatures in *Drosophila melanogaster* cooled at 0.1 and 0.05°C min $^{-1}$ compared to those cooled at 1.0 and 0.5°C min $^{-1}$. The converse was the case for flight in this study. The threshold for flight in *E. balteatus* is much higher than those for survival and torpor, and hardening at this threshold may therefore operate in a different manner.

A considerable difference was observed between the sexes. At low temperatures, more females flew than males, and the female FT_{50} was consistently 1.8 to 1.9°C lower than the male ($p<0.05$), in both non-hardened and hardened flies. Hardening acted to the same degree on both sexes, providing each with approximately 2.5°C of 'additional cold hardiness' at the flight threshold. This suggests that the hardening mechanism is the same for both sexes. Higher overall flight in females compared to males may be due to differences in the mechanisms of warming or cooling during flight (whether thermoregulatory or passive in nature) between males and females. These processes are known to be influenced by mass in other insects (Harrison and Roberts, 2000), and male *E. balteatus* were found to be consistently larger than females (see Chapter 2, Table 3.3). Evaporative cooling rates can significantly lower body temperature relative to ambient air temperature in hoverflies (Bressin and Willmer, 2000). In nature, the daily behaviours of

the two sexes are different: while males remain primarily in 'leks' near aphid colonies, either hovering or resting on foliage, females feed more and forage much more widely (Gilbert, 1984). Most studies of cold hardiness do not separate the sexes, but in some species, such as *E. balteatus*, differing body size and sex-specific behaviours may result in subtly different cold-hardiness profiles, which may be worthy of differentiation.

The effect of temperature on insect flight is clearly complex in nature, not least through interactions with other environmental factors and species-specific variation in thermoregulation (Harrison and Roberts, 2000). The mechanisms by which cold-hardening protects behavioural functions in insects are less well understood than for lethal injury, but may be no less important (Wang *et al.*, 2003; Powell and Bale, in press). Lethal temperatures may be only rarely encountered for most of the year, whereas movement thresholds may be encountered daily. Insects fly to forage, find mates and oviposition sites, avoid predators, defend territories, and for dispersal and migration. The constraint placed on flight by temperature is thus significant for many other insect behaviours, and RCH at the flight threshold may enable their expression at lower ambient temperatures.

Chapter 5

Rapid cold-hardening and chill coma in *Episyrphus balteatus*

5.1 Introduction

The historical focus of cold-hardening (and heat-hardening) research in insects has been on the lethal effects of temperature extremes. However, temperature is a limiting factor in many aspects of insect life history, and the importance of hardening at activity thresholds is less well understood than at lethal thresholds. The low-temperature flight threshold has been discussed in the previous chapter, but in species or life stages that do not or cannot fly, the threshold at which insects enter chill coma is equally important, limiting the ability of insects to forage, avoid predators, seek out preferred refugia and find mates.

The temperature at which insects enter chill coma, and the time taken to recover from it, are known to be reduced by acclimation and/or rapid hardening in certain species. It is necessary to distinguish between commonly used laboratory measures of chill coma temperature: (1) the temperature at which walking ceases (usually measured as distance walked in a given time, or temperature at which individuals cannot be made to move by the investigator), and (2) the temperature at which all muscle control is lost and the insect falls from a surface, usually termed the 'critical thermal minimum', CT_{min}. Insects may cease to walk at a temperature considerably above the CT_{min} , and though the two may be related, they should properly be considered as separate thresholds, although the term 'torpor temperature' is sometimes used for both. This study is concerned with the CT_{min} of *Episyrphus balteatus*.

Meats (1973) described a continual, rapid reacclimatization of torpor temperature (at which individuals could no longer be made to move) during cooling at rates up to 1°C/min in the fruit fly *Dacus tryoni*. In *Drosophila melanogaster*, cooling at ecologically-realistic rates decreased CT_{min} by 1.9°C (Kelty and Lee, 2001). Powell and Bale (in press) also found that rapid cold-hardening (RCH) decreased mean CT_{min} in the grain aphid *Sitobion avenae*, by up to 2.8°C, while long term acclimation at 10°C lowered the torpor temperature by only 0.7°C.

The time taken to recover from chill coma is also a useful measure of cold hardiness, and laboratory cold-selected lines of *Drosophila melanogaster* have faster recovery times (Anderson *et al.*, 2005), as do flies from geographical populations at higher latitudes: this has been shown in *D. melanogaster* (Hoffmann *et al.*, 2002; Ayrinhac *et al.*, 2004), *D. serrata* (Hallas *et al.*, 2002), and in *D. subobscura*, where the difference between tropical and temperate populations was only evident during recovery at low temperatures: at higher temperatures, temperate flies had no further advantage (David *et al.*, 2003). Also, sex differences have been recorded, with faster recovery times in females of *Drosophila melanogaster* (David *et al.*, 1998). However, when looking for the effect of RCH, Rako and Hoffmann (2006) found that treatment (18h at 0°C, or 4h at 4.5°C) did not reduce chill coma recovery time in *D. melanogaster*.

RCH of the chill coma temperature is likely to operate via similar mechanisms to those preventing chilling injury at otherwise lethal temperatures, such as the maintenance of cellular membrane fluidity (Lee *et al.*, 2006), perhaps due to increases in unsaturated phospholipid fatty acids (Overgaard *et al.*, 2005). However, some of the mechanisms by which an insect enters chill coma may be different from those causing chilling injury. The

similarity of the chill-coma recovery response to that of recovery from anaesthesia (such as that induced by CO_2) may imply that modification of the nervous system is involved (David *et al.*, 1998). Wing-muscle action potentials fall with temperature in *Apis melifera* and *D. melanogaster*, and variation in the ability to maintain them may contribute to species differences in coma temperature (Hosler *et al.*, 2000).

In the laboratory, chill coma recovery time decreases with decreased time spent in coma, and increased recovery temperature. However, the relationships are not necessarily linear. In *D. melanogaster*, as recovery temperature decreased, recovery time first increased only slightly and reached a plateau, before then increasing sharply (Macdonald *et al.*, 2004). This biphasic response has also been described in *D. subobscura*, and may be explained by the operation of two recovery mechanisms, the first operating at low stress levels, and the second becoming effective at increased stress levels (David *et al.*, 2003). Recovery from heat-coma in *Drosophila* species may involve the expression the heat-shock protein Hsp70 (Hoffmann *et al.*, 2003) but whether heat-shock and cold-shock processes are analogous is very much open to debate (Sinclair and Roberts, 2005). The work described in this chapter examines the effect of RCH on chill coma temperature (CT_{min}) and recovery time in *E. balteatus*.

5.2 Aims

- Determine the chill coma temperature of non-hardened flies, cooled at both rapid and ecologically realistic rates.
- Test the effect of RCH treatment on the chill coma temperature.

- Test the effect of a hardening treatment on chill coma recovery time.

5.3 Methods

Eggs were collected in 24h batches, and reared through to pupation. From each batch, adults emerged over a 2-5 day period. Newly emerged adult flies were collected at daily intervals and provided with water, sugar and pollen while awaiting use in experiments, which took place 1-2 days after emergence.

5.3.1 Chill-coma temperature (CT_{min})

To determine the temperature at which individuals enter chill coma (CT_{min}), adult *E. balteatus* were placed in a vertical glass column after the method of Huey *et al* (1992) (Figure 5.1). Coolant is pumped through the outer jacket of this column by a programmable refrigerated bath, allowing manipulation of the column's interior temperature. The temperature of the inner glass surface was monitored by an independent thermocouple device Flies were cooled from 20°C at a given rate and an observer recorded the temperature at which each individual fell out of the column. A glass dish was placed below the column to collect the flies as they fell.

During cooling, a number of individuals would fly out of the column, and were clearly not in torpor on landing in the dish. For this reason, an individual was only recorded as being in chill coma if: (1) on landing on its back, it did not immediately right itself, even if some leg movement was visible; or (2) on landing right way up, it did not immediately spread out its

legs and stand. Active flies (at circa 20°C) were observed to turn over and stand in this way. Occasionally, falling flies would knock others down; when this occurred, only one CT_{min} was recorded.

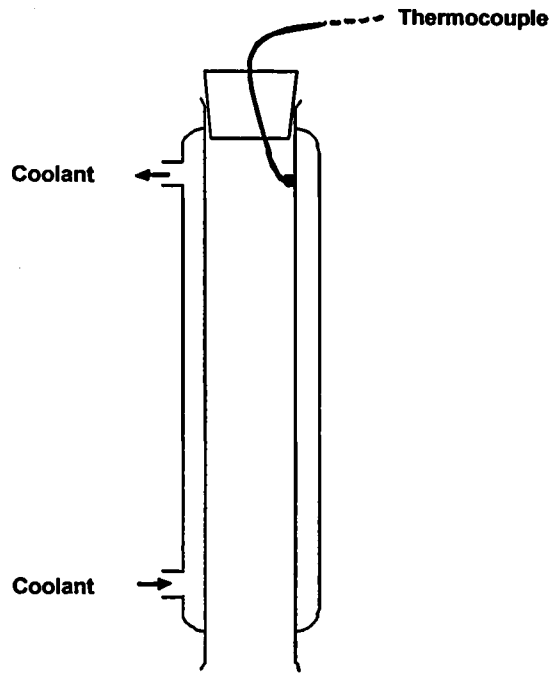

Figure 5.1 Controlled temperature column: apparatus used to determine chill-coma temperature in adult *Episyrphus balteatus*.

Males and females were treated independently. Ten flies were placed in the column per cooling run, and for most treatments, the procedure was repeated until the CT_{min} of at least 30 individuals had been recorded, usually requiring 4 or 5 runs. For the 0.1°C min^{-1} treatment, many more runs were required as more individuals flew out of the column. For this reason, a minimum sample size of 15 was used.

Within each treatment, the means of each run were compared by ANOVA. Only runs for

which there was no difference ($p>0.05$) were used, and the data then pooled by treatment. Variable condensation levels meant that a number of runs were discarded in this way. Treatment means were then compared by ANOVA and differences identified by Tukey's pairwise comparisons, maintaining family error rate of 0.05.

Treatments were as follows:

(1) In-column cooling rates of 1.0, 0.5 and 0.1°C min^{-1} were compared (cooling from 20°C to CT_{min} at that rate).

(2) RCH pre-treatment: Samples were first placed in boiling tube plugged with cotton wool and the following treatments were applied in a refrigerated bath:

   (a) 0°C, for 10, 60 or 120 min,

   (b) 10°C, for 10 or 60 min,

   (c) Cooling from 20 to 10°C at 1.0 or 0.5°C min^{-1}

The flies were then placed in the column at 20°C and 10 min were allowed for recovery, before cooling at 1.0°C min^{-1} to CT_{min}.

5.3.2 Chill-coma recovery time

To determine the length of time taken to recover from chill coma, individual flies were chilled in ice-water for 4 min (previously determined as the minimum time required for all flies to enter coma). They were then placed in a glass beaker suspended in a water bath. The temperature of the inner surface of the base of the beaker was monitored with a thermocouple and maintained at a set temperature by adjustment of the water bath. The flies were turned gently onto their backs and moved away from the sides of the beaker if necessary. The time

taken for an individual to right itself was recorded.

Recovery time in non-hardened flies was recorded at 10, 20 and 30°C. To test for RCH, flies were first held at 10°C for 60 min, before chilling in ice-water for 4 min to induce coma and then recording recovery time at 10°C. This hardening treatment was selected as it had previously been shown to induce RCH at the low-temperature threshold for flight in *E. balteatus*. Males and females were treated independently. Sample size was 20 to 30 per temperature and treatment.

Recovery times were decimalised and tested for normality by the Anderson-Darling method. All but one group showed significant non-normality, so decimal times were normalised by log-transformation, before testing with two-way ANOVA and Tukey's multiple comparisons.

5.4 Results

5.4.1 Chill coma temperature

In-column cooling rate

The results of the CT_{min} experiments are presented in Figure 5.2. In flies cooled at 1°C min^{-1}, the mean CT_{min} was 0.12 ± 0.2°C in males and 0.7 ± 0.3°C in females. CT_{min} varied significantly with in-column cooling rate ($p<0.01$), and there was no difference between the sexes across treatments. There was, however, a significant interaction between sex and cooling rate ($p<0.01$): cooling at 0.5°C min^{-1} decreased female CT_{min} but increased male

CT_{min}. Both male and female CT_{min} were highest when measured by cooling at 0.1°C min^{-1}, with means of 1.9 ± 0.5°C for males and 1.7 ± 0.5°C for females.

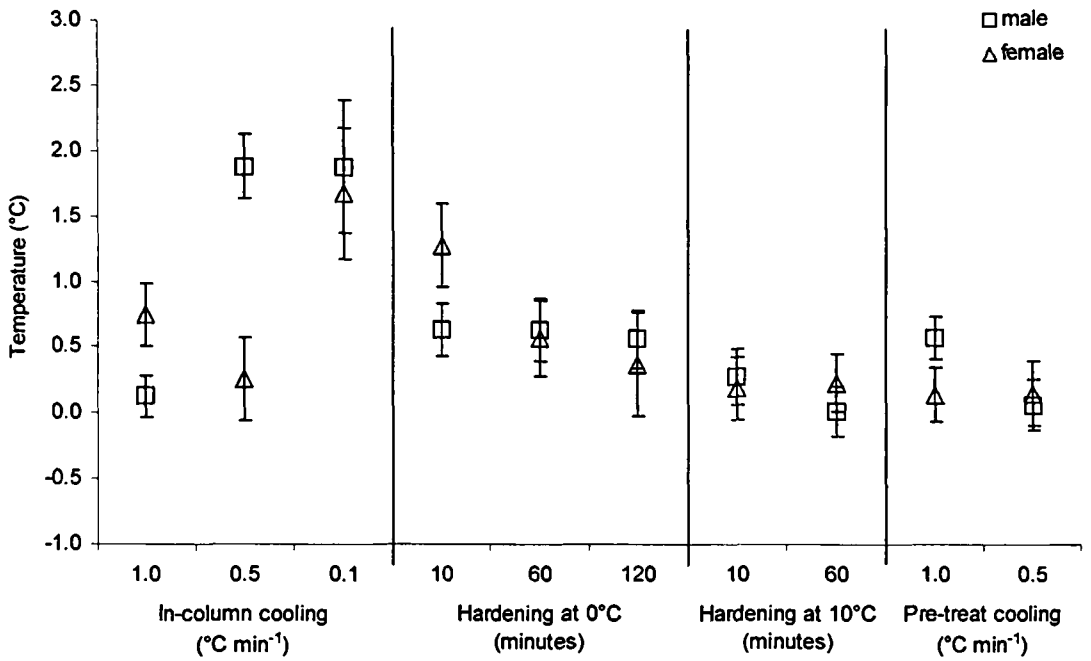

Figure 5.2 Chill-coma temperature in adult *Episyrphus balteatus*. Effect of in-column cooling rate and various hardening treatments. Error bars show SEM.

Effects of hardening treatments

The mean CT_{min} of flies cooled at 1°C min^{-1} without prior hardening treatment was tested against those for the different hardened groups. Although there was a slight increase in mean CT_{min} of both males and females following hardening at 0°C for 10min, none of the hardening treatments resulted in a statistically significant change in CT_{min} over that of flies cooled without prior hardening treatment. With increasing duration of hardening at 0°C for

10 to 120 min, mean CT_{min} of both males and females declined slightly, but again this change was not statistically significant. There was no difference in effect between the two durations of hardening at 10°C, or between the different pre-treatment cooling rates. In flies hardened at 0°C for 10 min, mean female CT_{min} was slightly higher than in males, but in all of the treatment groups there was no significant difference between the sexes.

5.4.2 Chill-coma recovery time

The mean recovery times at 10, 20 and 30°C are shown in Figure 5.3. Recovery time decreased significantly with increasing temperature ($p<0.01$). There was no difference between males and females at any temperature. Pooling males and females, the mean ± SEM recovery times were 1 min 29 sec ± 17 sec at 30°C, 3 min 38 sec ± 22 sec at 20°C and 7 min 27 sec ± 33 sec at 10°C.

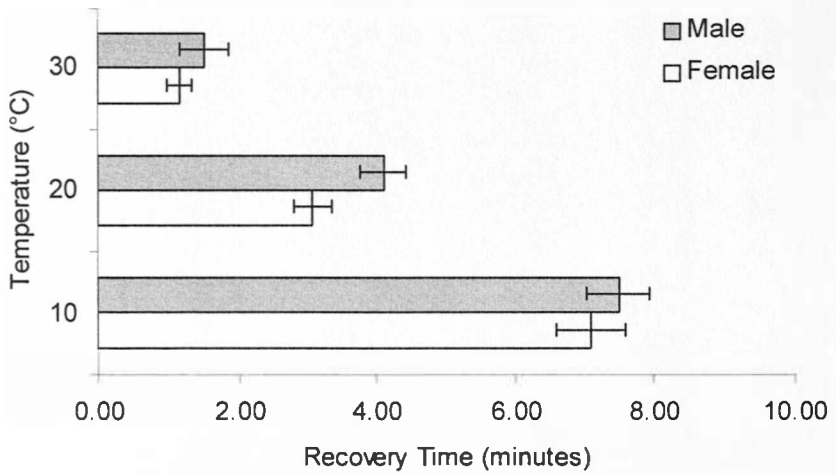

Figure 5.3 Time taken for adult *Episyrphus balteatus* to recover from chill coma at 10, 20 or 30°C. Error bars show SEM.

The mean recovery time of hardened flies (sexes pooled) was 4 min 52 sec ± 48 sec. This was significantly faster than in non-hardened flies ($p<0.05$) (see Figure 5.4; 'Non-hardened' data same as Fig. 5.3 '10°C'). Again, there was no difference between the sexes. However, it was noted that many of the hardened flies were not fully in coma after the 4 min ice-water stage used to induce torpor, and it was these individuals that had the fastest recovery times.

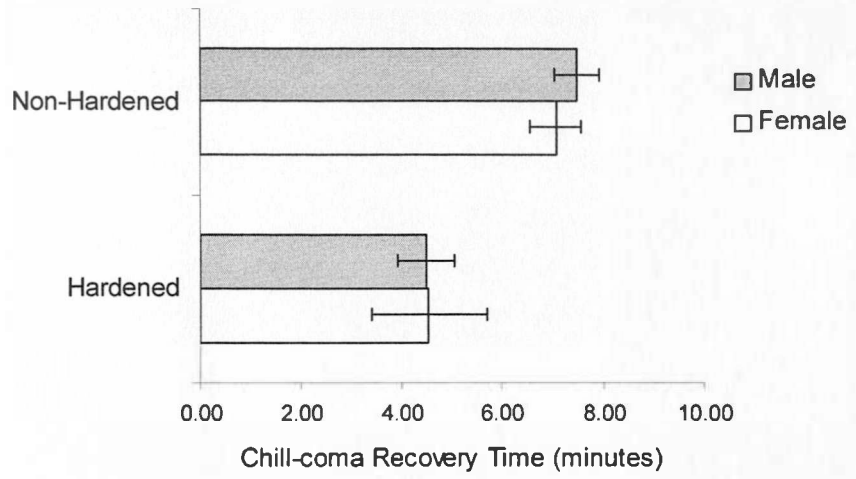

Figure 5.4 Effect of hardening on chill-coma recovery time in adult *Episyrphus balteatus*: recovery at 10°C. Error bars show SEM.

5.5 Discussion

The temperature at which an insect enters chill coma is not necessarily equivalent to the temperature at which it is unable to move. The threshold for movement may lie several degrees above the coma temperature (Mellanby, 1939). However, the CT_{min} measured by the glass column method is not entirely without ecological relevance in that it represents the

threshold below which the ability of an individual to maintain its position is lost. In some species, or individuals at certain times, the position adopted during periods of low temperature, for example at night, may be within a cryptic location, or thermally buffered microhabitat. Hardening at this threshold may therefore be useful, and several previous studies have shown that RCH can depress the CT_{min} by several degrees centigrade. However, hardening did not act to reduce CT_{min} in *E. balteatus*, whether as a pre-treatment, or simply by cooling to CT_{min} at a slower rate. It is possible that hardening gained by the pre-treatments could be lost on return to 20°C for 10min before cooling to CT_{min}: the (minimum) recovery period necessary to enable the flies to wake from coma and take up a standing position within the glass column. Such loss of hardening on return to normal temperatures is known to occur in other species hardened at around 0°C (Coulson and Bale, 1990). This loss generally occurs over a period of hours, rather than 10min, but the possibility of this occurring here cannot be discounted. However, this would not occur during the in-column cooling rate experiments, in which there was no recovery period.

Of the CT_{min}'s determined for *E. balteatus*, those of individuals cooled at the more 'ecologically-realistic' rate of 0.1°C min^{-1} are likely to be the closest to that experienced in nature. Lower CT_{min} after cooling at the fastest rate (1°C min^{-1}) may reflect the decreased length of time spent at temperatures close to the CT_{min} when cooling at this rate. It has already been shown that 0°C may represent a significant stress, to males at least, reducing flight proclivity, while 10°C induces hardening (see Chapter 4). When cooling at 0.1°C min^{-1}, 10 min is spent between 2 and 3°C (1°C above mean CT_{min}). However, when cooling at 1°C min^{-1}, individuals experience this temperature zone for a much shorter time (1 min).

The processes involved in recovery from chill coma are not well understood. Chill-coma recovery time in *E. balteatus* was significantly shorter at higher temperatures, supporting the conclusion of other studies that recovery is an energy-requiring process (Rako and Hoffmann, 2006). Indeed, there was an approximate halving of recovery time with each increase of 10°C, suggesting a classic Q_{10} response associated with an underlying biochemical process.

Although there is good evidence for reduced chill-coma recovery time as an adaptation to environments with lower temperatures (Hoffmann *et al.*, 2002), previous studies have not found brief RCH acclimations (less than 18h) operating on chill-coma recovery (Rako and Hoffman, 2006). While the data for *E. balteatus* appear to show that hardened flies recovered significantly faster, it was observed that many individuals in this group were not in full chill coma after 4 min in ice-water, while non-hardened individuals were. For this reason, it is not possible to conclude that RCH reduces recovery time *per se*. Instead, RCH may reduce the knock-down time at 0°C to less than 4 min, such that hardened individuals required significantly less time to recover. The difference between these two mechanisms may be of little importance to the insect, given that the effect was to reduce the negative effects of short-term exposure to 0°C. However, this result illustrates the need to integrate both temperature and time in consideration of chill-coma processes in insects, and *E. balteatus* may provide a useful model in which to do so.

Chapter 6

Relationships between phenology, abundance and temperature in the occurrence of *Episyrphus balteatus* in a suburban garden in the UK

6.1 Introduction

The importance of temperature in determining the phenology and abundance of insects has been be widely characterised, with examples from many taxa. As ectotherms, the life history of insects is limited by temperature at all levels, not least in their development. Many species' phenology is thus a response to changing temperatures through the year. The development of seasonally-acquired cold hardiness is an aspect of this, with many species developing through the summer to a cold-hardy, overwintering stage, and timing emergence to the onset of warmer weather and emergence of the food plant or prey.

For example, the effect of temperature on aphid phenology has been well characterised, and models developed that accurately predict the onset of aphid dispersal in the spring based on winter low temperatures (Zhou *et al.*, 1995). These models allow predicted flight dates to be made available to UK farmers each year.

In many species, the influence on population abundance of reproductive success in previous generations means that temperatures to which earlier generations were exposed may influence subsequent generations, and this may vary according to the particular life history and voltinism of the species concerned. For example, in a study of UK butterflies, greater abundance was associated (in some cases) with higher current-year June

temperatures in bivoltine species, higher previous-year Summer temperatures in species that overwinter as adults, and lower previous-year Summer temperatures in species of moist or semi-shaded habitats (Roy *et al.*, 2001).

Phenology also varies spatially, linked to differences in temperature across species' ranges. For example, in the UK, many species of butterfly appear later in the cooler East and North of their ranges, than in the warmer West and South (Roy and Asher, 2003).

Factors other than temperature affect phenology, particularly photoperiod, which is a dominant cue for seasonal change in many insects, interacting with temperature to determine phenology (Bale *et al.*, 2002). The spring emergence of many species shows synchronicity with food plant growth. For example, the onset of spring migration of the damson-hop aphid *Phorodon humuli* can be predicted accurately by a model incorporating both temperature and the onset of flowering in host plum species (Worner *et al.*, 1995). A particularly intriguing possible correlate of insect phenology is found between Batesian mimics and their models. Many species of hoverfly are considered to be Batesian mimics of Hymenopterans, and there is some evidence that the phenology and abundance of the most accurate mimic species may correlate with that of their models (Howarth and Edmunds, 2000). However, this is not thought to be the case in *Episyrphus balteatus*, which is a less specific mimic and is often much more abundant than its possible Hymenopteran models.

The relationship between temperature and phenology has a special case in migratory species, where the arrival of Spring migrants at a given location may depend more on temperatures in their area of origin than on local temperatures. For example, the migration

to the UK of various Lepidopteran species is linked to temperatures in France (Sparks *et al.*, 2005).

The importance of temperature in determining insect phenology has given this area of research a new vigour in recent years, as the effects of climate warming become ever more apparent and the value of long-term phenological datasets in understanding these processes becomes realised (Sparks and Menzel, 2002; Walther *et al.*, 2002).

One of the key currently-observable effects of climate warming is changes in species ranges, particularly northward expansion, as has been observed in many species of European butterfly (Parmesan *et al.*, 1999; Hill *et al.*, 2002) and UK Odonata (Hickling *et al.*, 2005). There is less evidence for similar loss of range at the low-latitude boundary, but this has been identified in some species, including a number of UK butterflies (Franco *et al.*, 2006). Similar range changes have been observed along altitudinal gradients. For example, in some species occurring at high elevations, the lower range boundary may increase in altitude, resulting in an overall decrease in range area. This has been shown in a number a butterfly species in Spain (Wilson *et al.*, 2005).

However, changes in phenology associated with climate warming have also been recorded, with the emphasis on advances in Spring events and the lengthening of activity periods and growing seasons (Sparks and Menzel, 2002; Menzel *et al.*, 2006). This is particularly well documented in European insects, with significant advances in dates of first emergence and peak abundance observed in many species, especially butterflies, over recent decades (Pollard, 1991; Roy and Sparks, 2000; Gordo and Sanz, 2006). An example of a typical result is provided by the Purple Emperor butterfly *Apatura iris* in

Switzerland, where Spring emergence has advanced by an average of 9 days in males and 12 days in females per decade since 1982 in response to increasing May temperatures (Dell *et al.*, 2005). In the Autumn, most trends appear to be toward delayed phenology, but the evidence is generally less clear than that for Spring events (Menzel *et al.*, 2006).

The principal manner in which such changes in phenology could threaten insect populations or species is by disrupting synchronicity with, for example, food plant phenology. For example, models have predicted that climate warming will induce earlier emergence of the winter moth *Operopthera brumata* in Scotland, but not change the timing of bud-burst in its host, the Sitka spruce, resulting in decreased synchrony between the two events, with implications for the abundance of the insect (Dewar and Watt, 1992). Buse and Good (1996) recorded egg hatch of *O. brumata* in relation to bud-burst of oak, under simulated conditions of climate change in experimental 'solardomes' (increased temperature and elevated CO2 levels), but did not find loss of synchrony. However, while bud-burst and larval hatch may become earlier by the same degree, maintaining synchrony, the brood size of insectivorous birds may be reduced because they cannot lay early enough to coincide with earlier peaks in abundance of moth larvae (Buse *et al.*, 1999). The effects of climate warming on phenological relationships between species, and in particular between trophic levels, may therefore be highly complex.

Further complexity in predicting phenological responses to general climate warming derives from the fact that warmer Autumns may have different effects to warmer Springs. For example, in an historical dataset of Lepidopteran phenology in the UK, high October temperatures correlated with delayed emergence the following Spring, whereas high temperatures in that Spring induced earlier emergence (Sparks *et al.*, 2006).

Understanding of species phenology, and the role of temperature as a determinant, is therefore of great current relevance, not only in application to agriculture through prediction of pest phenology, but also in understanding and predicting the effects of climate change, with implications for species conservation.

One of the key problems with studying insect phenology is the rarity of long-term datasets, particularly for autumn events, which may be harder to classify (Sparks and Menzel, 2002). Recording insect phenology and abundance over many years can have a number of inherent problems, not least variation in sampling effort. The establishment of national recording schemes has introduced greater standardisation in the recording of insect phenology at the national level, and enabled effective collation of large datasets. For many such schemes, the primary data source is observation while walking transects, and the datasets may show bias toward easily observed species (Dennis *et al.*, 2006). Modelling of animal abundance from transect-sampled data is prone to problems from variation in sampling effort, and violation of two assumptions in particular: that the probability of detection is the same for all individuals, and that all animals along the transect are detected (see Schwarz and Seber (1999) for review). The use of traps can provide more even sampling effort, but is not without uncertainties of its own. The relationship between the abundance of individuals in the sampling area and the number trapped may vary, or be unknown. Also, particular taxa may be more susceptible to capture than others, as may one sex. For example, in comparing the effectiveness of Malaise traps and coloured water traps at sampling Agromyzid flies in the same location, Schiers *et al.* (1997) found significant sex-bias in each method, with more males being caught in water traps, and more females in Malaise traps.

The work presented in this chapter is an analysis of a thirty-year dataset, recording the phenology and abundance of *Episyrphus balteatus* at a single site in the UK. The dataset is of unusual merit, in that it consists of weekly Malaise-trap catches, thus avoiding some of the pitfalls of transect sampling, and records the abundance of both males and females, providing a level of resolution unusual in such studies. The analysis presented below is the first to use the complete 1972-2001 dataset, and to relate temperature to the trends identified.

6.2 Aims

- Extract descriptors of phenology and abundance from the original dataset.
- Apply simple models to investigate the interdependence of these factors, and the influence of temperature on them.
- Compare the phenology of males and females.
- Identify any trends over time, and model these changes against a background of climate warming.

6.3 Methods

6.3.1 Phenological and abundance data

The original data consists of week-long Malaise-trap samples collected between 1 April and 31 October from 1972 to 2001, in a suburban garden in Leicester, UK (52°38'34N, 1°04'51W) (see Owen, 1981). The trap was sited in the same precise location each year. It

was emptied on the Sunday of each week (the sample *day*), and the count of male and female *E. balteatus* was recorded by week number, following the standard system whereby weeks run from Monday to Sunday, and week number 1 is that in which four or more of the first days of January fall. For the analysis presented in this chapter, the position of the sample days within the calendar year was standardised by transforming each sample date to a Julian day scale (1 = 1 January). As sampling began each year on 1 April, it was not necessary to allow for leap year data by adding an extra day after 28 February. Working with the data in this way also allows greater clarity for discussion, by dealing with calendar dates rather than week numbers.

Six descriptors were derived from the data for each year, four of phenology and two of abundance:

(1) date of first record,

(2) date of peak abundance,

(3) date of last record

(4) flight period (days from first record to last record),

(5) maximum weekly abundance,

(6) total annual abundance.

These were calculated for males, females and pooled (male + female) data. Pooling enables trends to be examined at the species level, and readily compared with other studies, most of which do not examine sex differences.

Where the maximum abundance value for one sex occurred in more than one week, the peak was taken as the week in which total (male + female) abundance was highest (occurred twice). If the maximum total abundance value occurred in more than one week,

the date of the median record was taken (occurred once). Where there was only one record for the year, the flight period was entered as 7 days (occurred once).

Most studies, particularly those considering large datasets at regional or national scales, measure flight period duration as the interval between first and last records. Some authors have suggested that the standard deviation of dates is a more useful indicator of flight period as it is less liable to distortion by single individuals living a week or more longer than the rest of their cohorts (Pollard, 1991). In the present study, the data for some years do contain significant outliers. However, in the use of a Malaise trap, a critical density of active insects is required before single individuals will be caught, and while that density is not known in this case, single records are likely to be representative of a larger active population. Furthermore, early occurrences of individuals is of interest in the study of advancing spring phenology, as unusually early occurrences may be a principal cause of changes in the population later in the summer. A sensitivity analysis was initially carried out using the data with outliers removed, and the principal trends identified were the same as those identified with outliers included. For these reasons, it was decided to not remove outliers from this dataset.

Because the data consist of week-long sample periods, any individual recorded on a given sample date may have been trapped on that day or any of the preceding 6 days. For this reason, mean phenology dates are presented below as the range of seven dates running up to and including the mean sample date. This sampling issue also means that any actual flight period can be either overestimated or underestimated by up to 6 days, but this source of error is equal in each year's dataset. Most insect recording schemes sample at weekly intervals, so this sampling effect is usual in phenological analyses.

6.3.2 Temperature data

The Central England Temperature (CET) is a standard index of monthly mean surface air temperatures in a region representative of central England (Manley, 1974; Parker *et al.*, 1992). The Leicester study site falls just outside this region, but the CET index has been shown to be representative of temperatures in other parts of UK (Duncan, 1991). The series runs from 1659 to the present day, and is maintained by the Hadley Centre of the UK Meteorological Office. The CET is considered a reliable and representative temperature index, and has been used by previous studies to model insect phenology and changes over time in the UK (e.g. Roy and Sparks, 2000). A consistent series of temperature records from a single recording station was not available in the local Leicester area for the study period 1972-2001.

6.3.3 Statistical analyses

Mean dates and abundances. The means of each descriptor were calculated and the differences between male and female means tested by paired t-tests, after confirming equal variance (F-test) and normality (Anderson-Darling). The abundance data were not normal, and so Levene's method (Levene, 1960) was used to confirm equal variances before normalising by log-transformation for the t-tests. The data for male flight period were also not normal, and no standard transformation was able to normalise them. It was therefore not possible to test the difference between the mean male and female flight periods.

Relationships between phenology and abundance. In order to determine whether phenology or abundance was influenced by events in the previous year, the datasets were tested for autocorrelation by simple linear regression of the phenology or abundance variable onto its lag (the value for the previous year). Further linear regressions were then conducted to identify any relationships between individual phenological descriptors (e.g. correlation between the date of first record and the total flight period). Finally, a stepwise regression procedure (Efroymson, 1960) was used to determine the effect of phenology on abundance, using first, peak and last record, and flight period, as the initial variables (further explanation of stepwise regression follows below).

Effect of temperature. The effect of temperature on phenology and abundance was modelled in two ways: (1) simple regression using mean annual CET as the descriptor; and (2) stepwise regression using monthly mean CET.

Trends over time. Trends in phenology and abundance over time were modelled by simple linear regression, using year as the predictor. Due to the non-normality of the abundance data, log-abundance was used. No change in abundance was found, and introduction of temperature terms did not increase significance, so abundance was not included in further models. Finally, phenology, temperature and time were brought together, regressing phenology onto year and either (1) monthly mean CET (by stepwise regression), or (2) mean annual CET. The relative fit and predictive ability of these two sets of resulting models was then compared.

Stepwise regression procedure (Efroymson, 1960). The timing of a phenological event may be affected by the temperature at the time it occurs, or the temperature of the

preceding month, or number of months, not necessarily in series. A stepwise regression approach was therefore used to relate changes in phenology and abundance to the mean monthly CET data. The months included were those including and prior to the month of the mean date of the event. For example, the mean first record of male *E. balteatus* fell in July. The months from January to July were therefore used as the initial variables in the model. The model was then allowed to include or reject any particular month, based on the significance of the correlation between the response variable and the temperature data for that month. The threshold significance level for inclusion or rejection of a term at each step was p=0.15, and the significance level for the model as a whole (including all terms) was p=0.05. The stepwise procedure was also used for the abundance data, but here the model could select from all months for the year.

General. All regression models were tested for fit (data subsetting lack-of-fit test), autocorrelation (Durbin-Watson procedure) and overall significance at p=0.05. The lack-of-fit and Durbin-Watson values are not presented, as only those models which were considered to have passed both tests were included in the results. All procedures were performed using Minitab (Minitab Inc, 2003).

6.4 Results

The total number of weekly records was 444, and the total number of flies recorded was 9413 (5356 males and 4058 females). The results for pooled data (male + female) are presented in the tables that follow, for species-level consideration and comparison with other studies. However, the patterns in the pooled data mostly reflected a balance of the

trends shown in the male and female data. For this reason, the emphasis in the following sections will be on the comparison of males and females.

6.4.1 Mean dates and abundances

In most years, flies were abundant from July to September, peaking in August, although there were generally some present in June, with occasional early and late records of individual flies, in April-May and October-November. This distribution is presented in Figure 6.1, with the mean monthly CET for each month.

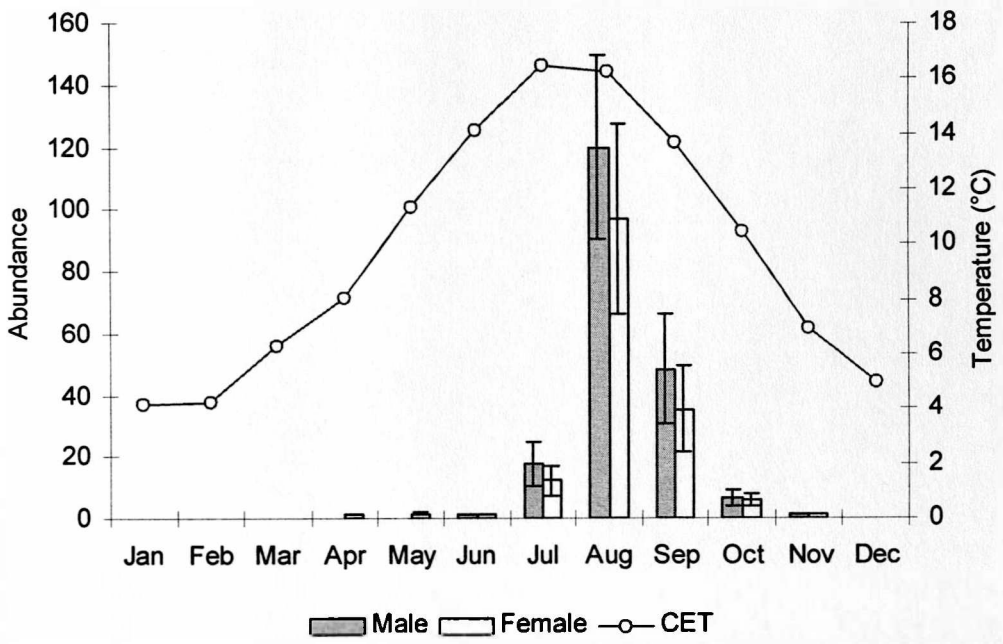

Figure 6.1 Mean monthly abundance of *Episyrphus balteatus* at the study site in Leicester, UK, 1972-2001, and mean Central England Temperature for the same period. Error bars show SEM.

The relationship between mean monthly abundance and temperature was significant for both males ($p<0.05$; R^2 37%), and females ($p<0.05$; R^2 36%). There was a very high level of year to year variation in the dates of first and last records, and the position of the peak record within the flight period (Figure 6.2). The dates of first and last record also varied in relation to each other, so that flight period was far from regular (Figure 6.3).

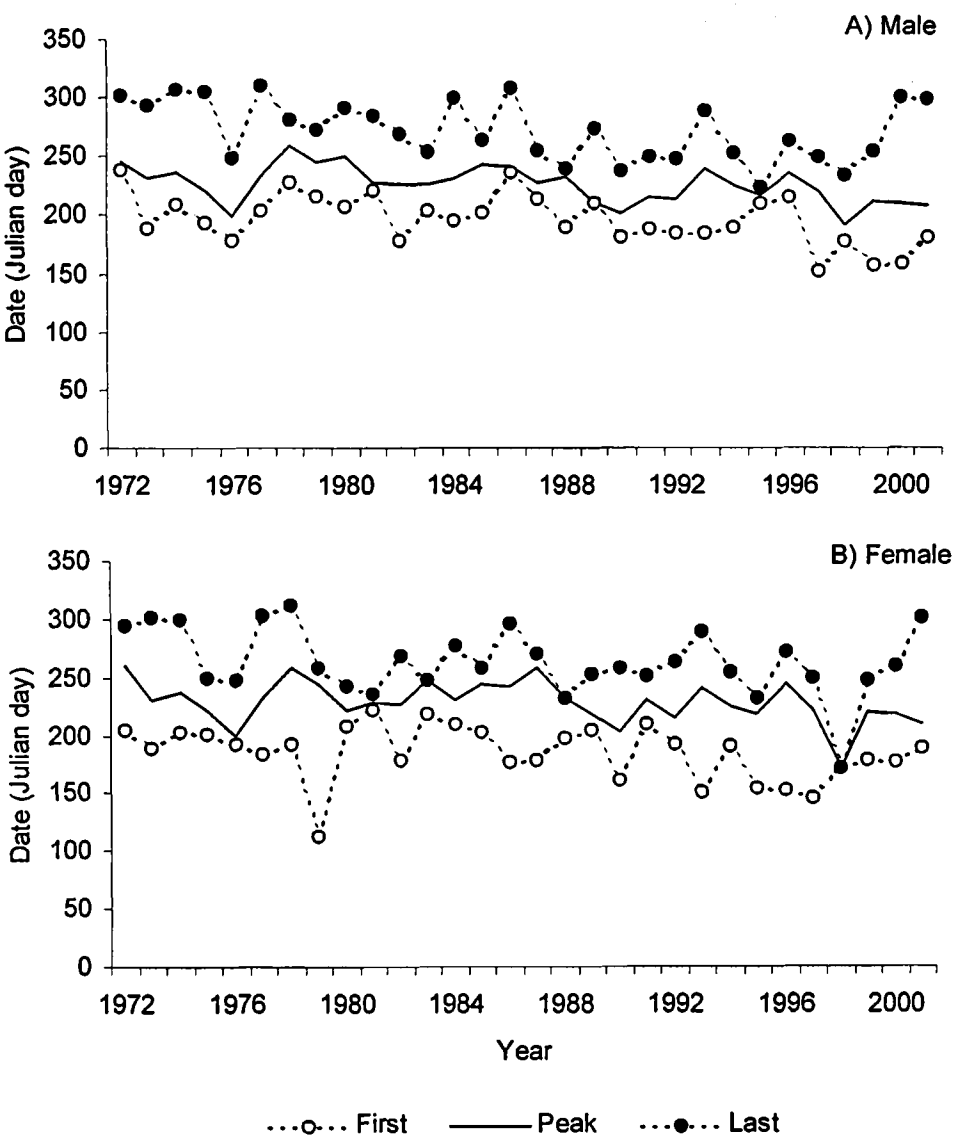

Figure 6.2 Variation in dates of first, peak and last record of *Episyrphus balteatus* over the period of the study: A) Males, and B) Females.

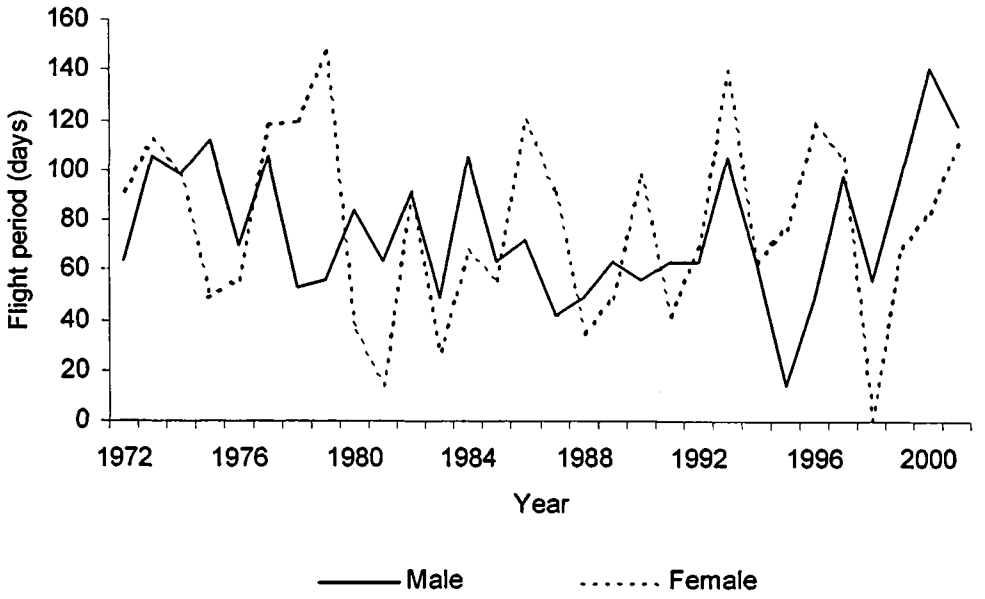

Figure 6.3 Variation in flight period of male and female *Episyrphus balteatus* over the duration of the study.

The mean dates of first, peak and last record are shown in Table 6.1. The mean first record was significantly earlier in females (4 July) than males (17 July) ($p<0.05$). However, the mean last record was significantly later in males (30 September) than females (20 September) ($p<0.05$). The mean flight periods were thus broadly similar, 75 ± 5d in males, and 79 ± 7d in females. Although it was not possible to test for difference between these two means, Figure 6.3 suggests that flight period was not consistently longer in one sex. The mean date of peak record was also not different in males and females, falling on 15 and 17 August respectively. Pooling the data for both sexes, *E. balteatus* was recorded on average from 30 June to 3 October, peaking on 13 August, with a flight period of 95d.

Table 6.1 Mean annual abundance, flight period, and dates of first, peak and last record, for male, female and pooled (male + female) *Episyrphus balteatus*. Within any one row, male and female means followed by the same letter are not significantly different at p=0.05. The original data were week-long Malaise trap catches; the mean Julian day presented is derived from the dates on which the trap was emptied at the end of each week. The true mean may therefore lie up to six days earlier, and the mean calendar dates above are therefore presented as a range of 7d, the latter of the two dates being that of the mean Julian day.

		Male	Female	Pooled
First record	Julian day ± SEM	198 ± 3.9 a	185 ± 4.5 b	181 ± 4.3
	Date range	11 Jul - 17 Jul	28 Jun - 4 Jul	24 Jun - 30 Jun
Peak record	Julian day ± SEM	227 ± 2.9 a	229 ± 3.4 a	225 ± 3.1
	Date range	9 Aug - 15 Aug	11 Aug - 17 Aug	7 Aug -13 Aug
Last record	Julian day ± SEM	273 ± 4.7 a	263 ± 5.3 b	276 ± 4.7
	Date range	24 Sep - 30 Sep	14 Sep - 20 Sep	27 Sep - 3 Oct
Flight period (days ± SEM)		75 ± 5.2	79 ± 6.8	95 ± 5.7
Maximum abundance ± SEM		83 ± 19 a	61 ± 17 b	143 ± 35
Total abundance ± SEM		179 ± 34 a	135 ± 33 b	314 ± 69

Abundance also varied greatly from year to year, both in terms of annual total and maximum weekly abundance, with each measure approximately co-varying (Figure 6.4). The maximum number of *E. balteatus* recorded in any one year was 1,467 in 1997 (with 837 recorded in a single week), and the minimum was just 4, recorded the following year, 1998. In most years, more males were recorded than females, and this was reflected in the mean annual (total) abundance, which was significantly higher in males (179 ± 34) than females (135 ± 33) ($p<0.05$). The maximum abundance in any one week was also significantly higher in males ($p<0.05$).

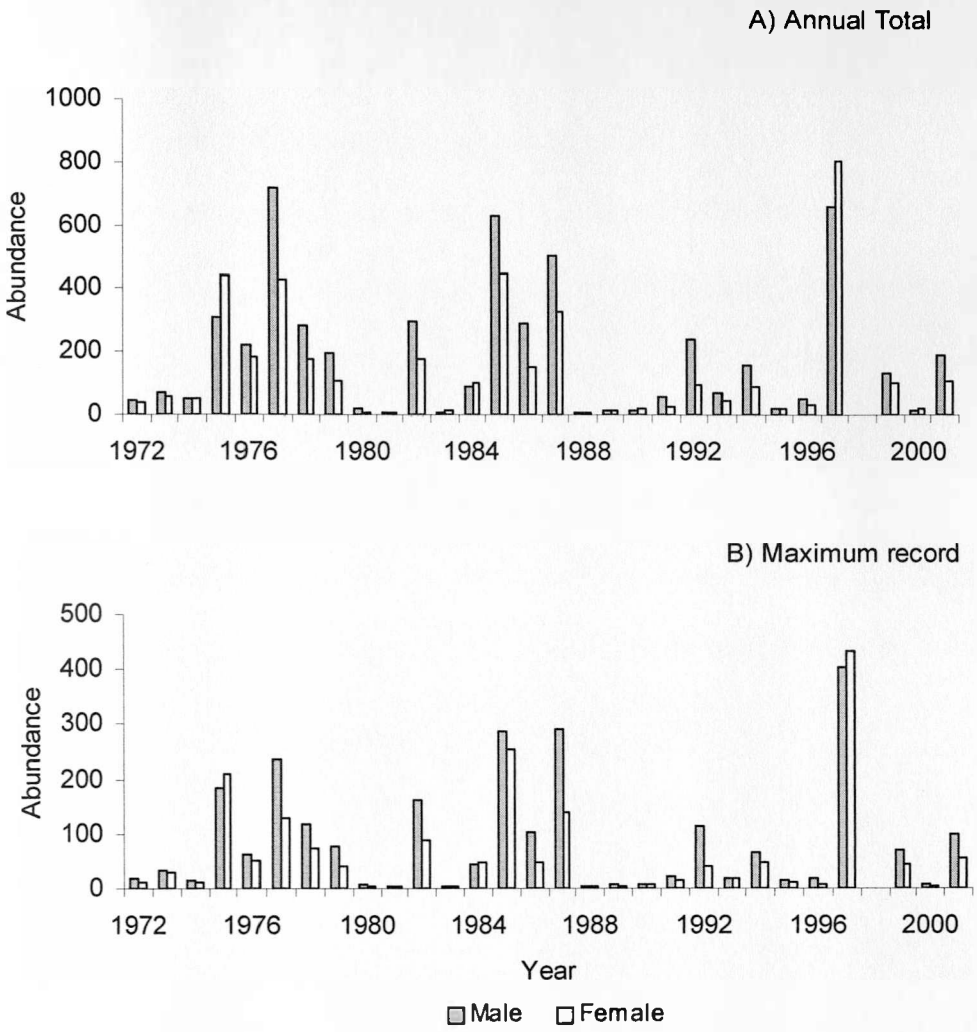

Figure 6.4 Variation in abundance of *Episyrphus balteatus* trapped over the period of the study: A) Total annual abundance, and B) Maximum record for one week. Note different y-axis scales.

6.4.2 Relationships between phenology and abundance

Autocorrelation was identified in only one variable, the date of peak record in males, which tended to be slightly later than in the previous year (lag coefficient 0.46, R^2 22%, $p<0.05$). This autocorrelation was monitored in all further regressions including this variable by use of the Durbin-Watson procedure, but was not found to be influential.

The results of the regressions of the phenological descriptors onto each other are presented in Table 6.2. The position of the peak record within the year was modelled by simple regression onto the date of first record, date of last record, and flight period. In males, the date of peak record was positively correlated with that of both first and last record, and did not change with flight period. In females however, the date of peak record was strongly correlated with the date of last record, but was independent of first record. Also, female peak record occurred slightly later in years with long female flight periods. The date of last record did not show a relationship to the date of first record in either sex. Not unexpectedly, duration of flight period in both sexes was strongly correlated with the date of first and last records, being shorter when the first record was later and last record earlier.

Table 6.2 Interrelationships between flight dates and period in *Episyrphus balteatus*. Table presents R^2, significance and predictor coefficient from regressions of response onto predictor.

	Male		Female		Pooled	
Response vs. predictor	R^2 (%)	coeff	R^2 (%)	coeff	R^2 (%)	coeff
Peak record vs. first record	31.1	0.41 **	0.3	0.04 ns	0.7	-0.06 ns
Peak record vs. last record	21.8	0.29 **	37	0.40 ***	16.2	0.27 *
Peak record vs. flight period	0	0.00 ns	18.6	0.23 *	14.8	0.21 *
Last record vs. first record	7.2	0.32 ns	0	0.02 ns	3.1	0.19 ns
Flight period vs. first record	25.9	-0.70 **	40.5	-0.98 ***	37.4	-0.81 ***
Flight period vs. last record	47.9	0.77 ***	57.6	0.98 ***	45.1	0.83 ***

No correlations were found between abundance and record dates. However, in females only, abundance was significantly correlated with duration of flight period (Table 6.3), but the coefficients were small, with an increase of approximately 1 individual per day of flight period.

Table 6.3 Correlation between abundance and flight period in *Episyrphus balteatus*. Results of stepwise regression of log abundance, using the logs of first record date, peak record date, last record date, and flight period, as the initial variables. In each case, flight period was the only predictor variable selected by the procedure. The table presents the R^2 and coefficients of log flight period, where significant.
ns p>0.05, * 0.05>p>0.01, ** 0.01>p>0.001, *** 0.001>p.

	Male		Female		Pooled	
Response vs. Log flight period	R^2 (%)	coeff	R^2 (%)	coeff	R^2 (%)	coeff
Log maximum weekly abundance	ns		31.0	0.92 **	ns	
Log total annual abundance	ns		43.7	1.09 ***	14.3	<0.01 *

6.4.3 Effect of temperature on annual phenology

There were strong correlations between temperature and phenology using both mean annual CET and monthly CETs as predictors. The results of regressing the phenological descriptors onto mean annual CET are presented in Table 6.4. There were no correlations between temperature and flight period. However, in males, the dates of first, peak and last record were significantly earlier in warmer years, by approximately 20 days per 1°C increase in annual mean temperature. The R^2 values were particularly high for annual CET as a predictor of date of first record (R^2 43%) and date of peak record (R^2 55%). In females, dates of peak and last record were also strongly correlated with temperature, particularly peak (R^2 46%). However, the date of first female record showed an absence of relationship with temperature.

Table 6.4 Correlations between phenology and annual mean CET in *Episyrphus balteatus*. Table presents R^2 and predictor (temperature) coefficients from simple linear regressions of the response onto annual mean CET. ns p>0.05, * 0.05>p>0.01, ** 0.01>p>0.001, *** 0.001>p

	Male		Female		Pooled	
Response	R^2 (%)	coeff	R^2 (%)	coeff	R^2 (%)	coeff
Date of first record	42.9	-25.3 ***	0.1	-1.57 ns	ns	
Date of peak record	54.7	-20.8 ***	46.4	-22.9 ***	40.8	-19.4 ***
Date of last record	19.2	-20.2 *	18.3	-22.1 *	19.7	-20.1 *
Flight period	ns		ns		ns	

A similar general pattern was found in stepwise regression using monthly CETs as predictors: in both sexes, peak and last record showed strong (negative) correlation with temperature, but first record did so only in males (Table 6.5) However, unlike the models using mean annual CET, the stepwise procedure identified an inverse relationship between flight period and temperature in females, though none in males.

Table 6.5 Correlations between phenology and monthly mean CET in *Episyrphus balteatus*. Summary of stepwise regressions of response, with monthly mean CET's as the initial predictor variables. Table presents adjusted R^2 and the months included in the final model (1=January). The sign of the coefficients with temperature for each is indicated before the month number (+/-).
ns p>0.05, * 0.05>p>0.01, ** 0.01>p>0.001, *** 0.001>p.

	Male		Female		Pooled	
Response	R^2 (%)	Months	R^2 (%)	Months	R^2 (%)	Months
Date of first record	45.9	-3,-4,-6 ***	ns		ns	
Date of peak record	63.4	-5,-7,-8 ***	57.8	-5,-8 ***	50.7	-5,-8 ***
Date of last record	31.2	-4,-5,-7 **	24.5	-1,-9 **	35.8	-3,-4,-7 **
Flight period	ns		29.3	-1,-9 **	22.0	-7,+10 *

The months identified as significant varied between models, and in no case were contiguous. For the date of peak record, August was included for both males and females. This is month in which the mean peak record for each falls. The mean date of last record

for both sexes occurs in September. However, the temperature for this month was only found significant in females. The latest month included in the model for males was July. For the date of first record (males only), June CET was found significant, the month prior to that in which the mean date falls. In all models, certain months were included that fall well before the event in question. This was most noticeable in the date of last female record, where the January CET was found significant. This occurred to a lesser extent in the other models, where Spring months were included.

In the pooled data, there was a significant correlation between flight period and mean CET for July and October. However, the two months did not show the same relationship, with higher July temperatures being associated with shorter flight period, and higher October temperatures being associated with longer flight period.

6.4.4 Effect of temperature on annual abundance

No correlations were identified between mean annual CET and abundance of *E. balteatus*. However, both maximum and total abundance correlated strongly with the temperature of certain months (Table 6.6). As with the stepwise regression models for phenology, significant relationships with monthly temperatures occurring well before the flight period were identified. Here, January was included in all of the models, and had the smallest p-value of any of the months included in each case. The maximum abundance of both males and females showed an inverse relationship with January CET, and a positive relationship with July CET. The total abundance of males correlated with January CET only, again an inverse relationship. Female total abundance correlated negatively with January CET, positively with July CET, and negatively with September CET.

Table 6.6 Correlations between abundance and monthly mean CET in *Episyrphus balteatus*. Summary of stepwise regressions of log abundance, with log monthly mean CET's as the initial predictor variables. Table presents adjusted R^2 and the months included in the final model (1=January). The sign of the coefficients with temperature for each month is indicated before the month number. ns p>0.05, * 0.05>p>0.01, ** 0.01>p>0.001, *** 0.001>p.

	Male		Female		Pooled	
Response vs. log temperature	R^2 (%)	Months	R^2 (%)	Months	R^2 (%)	Months
Log maximum weekly abundance	31.7	-1,+7 **	26.5	-1,+7 **	27.5	-1,+7 **
Log total annual abundance	26.8	-1 **	30.2	-1,+7,-9 **	23.2	-1 **

6.4.5 Trends in phenology and abundance over time

The preceding analyses show how temperature affects phenology in any one year. However, variation in phenology and abundance over the period of the study are shown in Figures 6.2 and 6.4 respectively. In order to model these changes, the descriptors were regressed onto year. No significant correlations were found with maximum or total abundance, suggesting that these variables did not change through the study period. However, a trend towards earlier record dates may be observed in Figure 6.2, and significant correlations were identified by the regressions (Table 6.7).

Table 6.7 Trends over time in phenology of *Episyrphus balteatus*, 1972-2001. Results of regressing response onto year. ns p>0.05, * 0.05>p>0.01, ** 0.01>p>0.001, *** 0.001>p.

	Male		Female		Pooled	
Response	R^2 (%)	coeff	R^2 (%)	coeff	R^2 (%)	coeff
Date of first record	34.7	-1.44 **	11.9	-0.96 ns	15.8	-1.07 *
Date of peak record	24.2	-0.88 **	16.9	-0.88 *	9.2	-0.59 ns
Date of last record	19.3	-1.28 *	13.3	-1.20 *	21.0	-1.32 *
Flight period	0.2	0.16 ns	0.3	-0.24 ns	0.5	-0.25 ns

In males there was a significant trend towards earlier dates of first, peak and last record through time. The year coefficients in the models of first and last record are similar, so the lack of correlation between flight period and year is logical. In females, the dates of peak and last record also showed significant negative correlation with year, and the coefficients for year were very similar to those for males. However, there was no correlation between date of first record and time in females, and also no correlation between flight period and time.

6.4.6 Trends in phenology and temperature over time

The mean annual CET also changed over the period of the study, increasing by approximately 0.3°C per decade when modelled by simple linear regression (Mean Annual CET = -50.0 + 0.0301year R^2 22.5% $p<0.01$). This trend is shown in Figure 6.5.

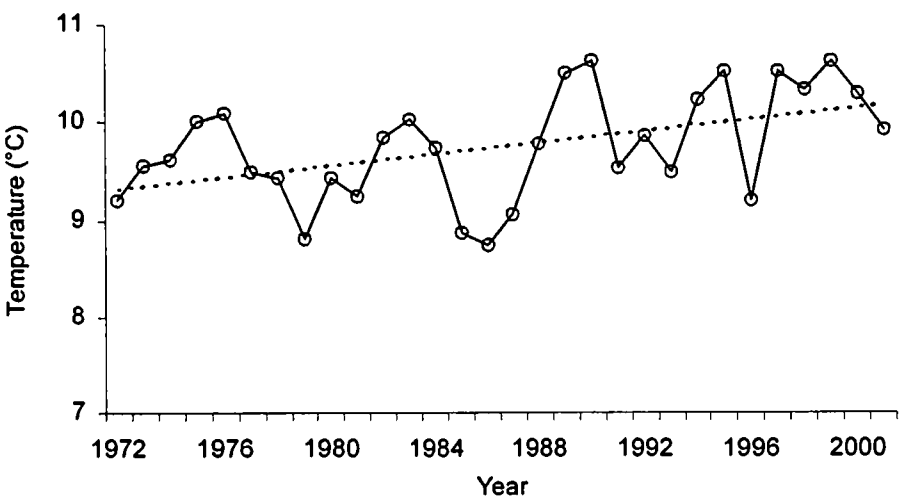

Figure 6.5 Increase in annual mean Central England Temperature over the period of the study, 1972-2001. Trend line fitted by least squares method.

The final series of models for *E. balteatus* therefore incorporated temperature, as mean annual CET or monthly CETs selected by stepwise regression, as well as a year term to account for changes over time. Where there was a significant relationship, these models mostly provided the best fit and highest R^2 values, and were therefore the most suitable for deriving a measure of the change in each phenological variable with every 1°C of warming. These results are presented in Table 6.8.

Table 6.8 Summary of regression models integrating phenology of *Episyrphus balteatus* with temperature and change over time. Response variables: date of first record, date of peak record, date of last record, and flight period (days). A: Using mean annual CET as the temperature variable (T). B: Using stepwise regression with monthly mean CET's as initial predictors (CET month number, 1=January). The sign of the coefficient for each term is indicated under Terms Included. Yr = year. A term in brackets indicates a coefficient value of zero. Values for change per +1°C are number of days (omitted for non-significant models). No model for the date of peak record in pooled data was sufficiently well fitted to be included. ns $p>0.05$, * $0.05>p>0.01$, ** $0.01>p>0.001$, *** $0.001>p$

	Male				Female				Pooled			
Response	Terms included	R^2 (%)	sig	Change (+1°C)	Terms included	R^2 (%)	sig	Change (+1°C)	Terms included	R^2 (%)	sig	Change (+1°C)
A												
First	-T,-Yr	49	***	-18.7	+T, -Yr	8	ns		+T, -Yr	12	ns	
Peak	-T, -Yr	54	***	-18.4	-T, -Yr	44	***	-21.1	-T, (Yr)	37	**	-19.4
Last	-T, -Yr	21	*	-13.6	-T, -Yr	16	*	-17.0	-T, -Yr	22	*	-13.3
Period	+T, +Yr	0	ns		-T, +Yr	4	ns		-T, +Yr	4	ns	
B												
First	-2, -Yr	47	***	-4.6	+1, -Yr	16	*	4.3	+1, -Yr	21	*	4.3
Peak	-5, -7, -8, -Yr	64	***	-14.6	-5, -8, -Yr	57	***	-16.4				
Last	-4, -5, -7, -Yr	31	***	-22.0	-9,-Yr	24	**	-12.3	-Yr	7	*	No term included
Period	+12, +Yr	3	ns		-1, -9, +Yr	28	**	-27.8	-1, -9, +Yr	7	ns	

Using annual mean CET as the temperature variable, the significant correlations were those already identified by regressing phenology onto annual CET without the year

variable (Table 6.8A, c.f. Table 6.4). Inclusion of the year term increased the R^2 values in the models of first and peak male record, but not in the other models. The significance levels were unchanged. Again, the models showed first, peak and last record of males all became earlier, whereas in females only peak and last records did so.

Including temperature in the stepwise regressions resulted in little change in R^2 or significance levels of the models compared to those not including the year term (Table 6.8B, c.f. Table 6.5). However, including the year term identified an additional significant correlation, between date of first record and temperature + year in females. This model identified increasing mean January CET as a significant correlate of later dates of first record. The other models for female record dates identified negative correlations with CET for the same months as did the models without the year term, except in that for date of last record, where January CET was no longer a significant predictor when the year term was included. Also, while no significant trend in flight period over time was identified independent of temperature (Table 6.7), the stepwise model of temperature and time showed a significant correlation in females, with flight period showing a positive relationship with year, but a negative one with January and September CET.

In males, the model for date of last record identified the same months' CET (April, May and July) as significant with or without the year term. In the model for date of peak record, May CET was added to July and August CET. In the model for date of first record, only February CET was significant, and March, April and June CETs were no longer included.

In the pooled data, no model was fitted for date of peak record. For the date of last record, no temperature term was included, but there was a negative correlation with year. Also, a significant model was fitted for first record becoming earlier with time, but later with increased January CET.

Across all the above models, the trend was for dates of first, peak and last record to become earlier over time and with increasing temperature, and for flight period to remain unchanged. Two exceptions were in females, where the models suggested that (1) date of first record was either unchanged, or became earlier over time and was positively correlated with January CET, and (2) flight period increased over time, but was negatively correlated with temperature.

6.5 Discussion

This dataset is unusual in that it consists of week-long trapping records. Most data used in phenological analyses of insects consists of observations, often on transects walked by observers on single days at weekly intervals, and as such can suffer from variation in sampling effort and effectiveness (Schwarz and Seber, 1999; Dennis *et al.*, 2006). This dataset for *E. balteatus* benefits from very consistent sampling effort, and by spreading its observation across a week's trapping is less vulnerable to false low counts on single days caused by poor weather (see Owen, 1981).

Year to year variation in phenology and abundance at a single location may not be representative of processes at a regional or national level (Schwarz and Seber, 1999). *E.*

balteatus occurs throughout most of the UK, but information on its spatial variability through time is limited, and may be patchy over short timescales (Stubbs, pers. comm.). Nevertheless, the monthly abundance pattern of *E. balteatus* recorded in the Leicester dataset closely matches that recorded for the species at the national level by the UK hoverfly recording scheme, although the national data show a greater abundance before June and after September (Ball and Morris, 2000).

Preliminary analysis of this dataset organised using a week number system identified many of the same trends as using the Julian day system, but model fit was generally weaker. This suggests that the use of the Julian day transformation to standardise the position of the dates of first, peak and last record within the year, was a successful method of increasing data resolution and consistency, as used in similar studies (e.g. Sparks and Carey (1995); Gordo and Sanz (2006)). It is necessary to be cautious in the interpretation of the models: correlation is not causation, and while some of the months identified by each stepwise regression may be true determinants of phenology, others may simply be co-variants (Bigg, 1953; Hocking, 1976; Stigler, 2005). This caution given, a number of significant trends are shown in the data, and certain relationships are shown by each of the different modelling approaches used.

Abundance was found to vary significantly with temperature: years of high abundance were correlated with low January temperatures and high summer temperatures. Increased abundance during warmer summers is not unexpected, as higher temperatures result in faster development rates in *E. balteatus* (Hart and Bale, 1997b), and increased abundance of aphid prey (Zhou *et al.*, 1997; McVean *et al.*, 1999; Cocu *et al.*, 2005). The correlation between low January temperatures and high abundance of *E. balteatus* is surprising, given

that the species is not present in the UK in January in significant numbers (Stubbs and Falk, 1983; Hart and Bale, 1997b; Ball and Morris 2000). However, this correlation might also operate via prey abundance. For example, low temperatures in January and February are associated with high subsequent abundance of the pea aphid *Acyrthosiphum pisum* in eastern England (McVean *et al.*, 1999). Abundance may also depend on synchronicity of phenology between predator and prey. For example, peak summer abundance of *E. balteatus* larvae in cereal crops in Germany was found to be lower when the first eggs were recorded soon after the onset of aphid infestation in the spring, whereas when the first *E. balteatus* eggs were not recorded until later (i.e. when the aphid population had grown considerably in size) the peak summer abundance was greater (Tenhumberg and Poehling, 1995).

Many species of insect are recorded earlier in warm years, such as *Apis mellifera* in Spain (Gordo and Sanz, 2006), various aphids in Europe (McVean *et al.*, 1999; Cocu *et al.*, 2005) and 35 species of UK butterfly (Roy and Sparks, 2000). The dates of peak and last record in *E. balteatus* were significantly earlier in warmer years, but early first records correlated with high temperature only in males. There was also no correlation between temperature and flight period, as identified in many UK butterflies (Roy and Sparks, 2000), but this may relate to voltinism (discussed further below). The migration of *E. balteatus* into the UK may also be affected by temperatures in the area from which migratory adults depart, and incorporation of climate data from other parts of the species' range could improve the accuracy of the models of *E. balteatus* UK phenology. For example, Sparks *et al.* (2005) showed that the UK phenology of various species of Lepidoptera is linked to temperatures in France, from where these species migrate to the UK.

Some insects show year to year autocorrelation in annual abundance or phenology, and this can vary depending on aspects of life history. For example, in a study of UK butterflies, greater abundance was associated (in some cases) with higher current-year June temperatures in bivoltine species, higher previous-year summer temperatures in species that overwinter as adults, and lower previous-year summer temperatures in species of moist or semi-shaded habitats (Roy *et al.*, 2001). It is not known what influence reproductive success in the UK has on the numbers of *E. balteatus* in the following spring (Rotheray, 1989). The timing of peak male abundance did show a significant linear relationship with the previous year's value, but, even here, the R^2 value of 21.5% suggested that only around a fifth of the variation in timing of the male peak might be explained by autocorrelation. In the absence of any obvious mechanism, it is difficult to conclude that autocorrelation is an important determinant here. Overall, the large year to year variation in the data, coupled with a general lack of autocorrelation in the time series, suggests that phenology and abundance one year have little influence on the following year, at this field site. This supports the conclusion of Hart and Bale (1997b) that *E. balteatus* does not overwinter in significant numbers in the UK.

The interrelationships between the dates of first, peak and last record, and consequently the flight period, may provide insight into the timing of generations in *E. balteatus* in the UK, a subject for which there is limited data (Hart *et al.*, 1997b). In both males and females, the date of last record correlated significantly with the timing of the population peak, with later peaks associated with later last records. The magnitude of the effect was approximately equal in both sexes. It is not known if the last individuals recorded represented the progeny of the population present at peak abundance, but the number of

days from peak to last record (46 in males, 34 in females) correlates well with the 30-day development time (egg to adult emergence) calculated by Hart *et al.* (1997b) in the laboratory at 15°C, and the 45-day average spring generation time reported by Ankersmit *et al.* (1986). The mean number of days from first record to the date of peak population abundance (pooled data) was also in this region (44 days), and it may be that the intervals from first to peak record, and peak to last record, represent the generation timings of *E. balteatus* in nature. However, it is interesting to note that, in *E. balteatus* males at least, earlier first record was associated with earlier peak record and no lengthening of flight period. This is the relationship identified for *univoltine* species by Roy and Sparks (2000), in a survey of 35 species of UK butterflies, whereas in multivoltine species, early first record was associated with a longer flight period and no change in peak record.

The analysis showed a number of differences between males and females. Males were generally more abundant than females, but it is not known if this represents the true population sex ratio or a bias in trapping effectiveness. Sex bias in the effectiveness of Malaise traps has been demonstrated in, for example, the trapping of Agromyzid flies (Schiers *et al.*, 1997), and trap colour has been shown to affect catches of certain hoverfly species (Wratten *et al.*, 1995). The mean dates of first record were, however, significantly different, with the first females of Spring being recorded on average 13 days earlier than the first males. The models also suggested that first female record was independent of temperature, while first male record was not. This may relate to the lower flight threshold identified in female *E. balteatus* (chapter 4). Schneider (1958) also trapped female *E. balteatus* earlier in the spring than males.

The general result of modelling changes in phenology and temperature over time was that the dates of peak and last record advanced over the thirty-year study period, as did the date of first record in males, but not females. The most conservative estimate of this advance was around 4 days per 1°C increase, although temperature did not explain all the variation in the phenological data. These results are consistent with advancing spring events identified in insects and other taxa (Sparks and Menzel, 2002; Walther *et al.*, 2002; Menzel *et al.*, 2006). For example, first record of a range of British butterfly species are predicted to advance by 2-10 days per 1°C increase in CET (Roy and Sparks, 2000). Evidence for changing autumn events is less clear, but indicates a trend towards a delay in autumn (Sparks and Menzel, 2002). This is in contrast to the advance in date of last record seen here in *E. balteatus*. While earlier spring events may be linked to warmer spring temperatures, earlier last record dates in *E. balteatus* are difficult to explain, given that warmer autumn temperatures might be expected to increase the flight period by allowing activity later into the year (Roy and Sparks, 2000; Bale *et al.*, 2002).

The models of phenology-change relative to temperature and year, suggest that the phenology of *E. balteatus* may change significantly with climate warming. The species is widespread and abundant across its range (Rotheray, 1989; Gilbert, 1993), and the results of this study suggest that flight period is not decreasing, a factor associated with low conservation threat in European hoverflies (Sullivan, 2000). However, effective predation of aphids in cereal crops has been shown to depend on synchronicity between aphid build-up and early oviposition by *E. balteatus* females (Tenhumberg and Poehling, 1995). Significant changes in the phenology of *E. balteatus* may thus have implications for its importance as a natural enemy.

Chapter 7

General discussion

7.1 Introduction

The study of insect cold hardiness has held a fascination for biologists for much of the 20th Century. Low temperature acclimation with respect to the chill coma temperature and the lethal threshold was described by Mellanby (1939). For many modern researchers, the development of the subject and many of its founding principles were laid out by Salt (1961). It is understandable that scientists are fascinated by the ability of insects to survive the harshest of low temperature environments, in some species by synthesising a range of cryoprotectants to prevent internal freezing by allowing supercooling, and in others by having developed the ability to withstand the partial freezing of fluids and tissues (Baust, 1973; Zachariassen, 1985; Duman, 2001). The seasonal response to changing climatic conditions has now been extensively characterised in both freeze tolerant and freeze avoiding species, with an increasing understanding of the associated physiological and biochemical processes and the structure-function relationships of the main cryoprotective compounds (ice-nucleating agents, polyols and antifreeze proteins) (see Bale, 2002, for review).

There have been a number of significant advances in understanding beyond the 'two strategy' view of cold hardiness. Firstly, Lee *et al.* (1987) described the process of rapid cold-hardening in studies on the flesh fly *Sarcophaga crassipalpis.* Both Mellanby (1939) and Meats (1973) made similar observations, but it was the Lee *et al.* (1987) paper that brought the phenomenon to the attention of today's researchers, and rapid cold-hardening

has since been described in the majority (though not all, see Chen *et al.* (1990)) of the species in which it has been investigated (Wang *et al.*, 2003). Secondly, Bale (1993) argued that while the existence of freeze tolerant and freeze avoiding species was not in doubt, if the purpose of each strategy was to prevent mortality at low temperatures, then very few of the species studied so far could be placed in either of these two categories. Rather, the majority of insects died at temperatures well above that at which they froze, the number depending on the temperature and duration of the exposure. This introduced three further classes of insect cold tolerance: chill tolerant, chill susceptible and opportunistic survival (Bale, 1996), and was seen as an attempt to introduce a degree of ecological realism into a subject that had been dominated by laboratory studies on a limited range of species, particularly those from polar or alpine environments.

7.2 Connecting physiology to ecology: an opportunity at behavioural thresholds

The need to link the physiology of insect cold tolerance to its ecological relevance was also recognised by Lee and co-workers in the studies that followed their 1987 description of rapid cold-hardening (Chen *et al.*, 1987; Czajka and Lee, 1990). A series of studies by different researchers, on a wide range of species, focused on the conditions inducing the response, and the limits of its action (Coulson and Bale, 1990, 1991; Kelty and Lee, 1999; 2001; Worland and Convey, 2001). For example, accepting that insects may not regularly experience a 30°C change in temperature in a matter of minutes, it has now been demonstrated that rapid cold-hardening can be induced by cooling at 'ecologically-realistic' rates, through an 'acclimation zone', generally between 10 and 0°C (Kelty and Lee, 1999, 2001; Kim and Song, 2000). Furthermore, the response does not apply only to species in which substantial mortality occurs at temperatures well above the freezing

temperature. Polar arthropods rapidly cold-harden by shifts in the SCP (Worland and Convey, 2001), a remarkable phenomenon, given that in the timescales involved, the organisms are unable to synthesise cryoprotectants, evacuate their gut, or reduce body water content to any great extent. Recently, rapid cold-hardening has also been found to increase the degree of freeze tolerance in *Belgica antarctica* (Lee *et al.*, 2005).

In some ways however, it seems that the continuing discovery of different biochemical compounds with clearly identified roles in freeze tolerance and avoidance, aided by increasingly sophisticated analytical techniques, and even the subsequent focus on the peculiarities of rapid cold-hardening, has diverted attention from other, equally important ecological realities (see Chown and Storey, 2006). One such example is found in highly mobile species that migrate or disperse over various distances, from summer feeding areas to parts of their range where winter temperatures do not become lethal. While this strategy has frequently been described as a mechanism of avoiding low temperatures in winter, it has been studied in very few species, other than the Monarch butterfly *Danaus plexippus* (Larsen and Lee, 1994; Troyer *et al.*, 1996). Furthermore, this dominant focus on the lethal effects of low temperatures has obscured the fact that there are other important thermal thresholds, particularly for basic movement (walking and flight), necessary to foraging, seeking out refugia, avoiding predators, and, ultimately, mate-finding and reproduction (Rogowitz and Chappell, 2000; Shreve *et al.*, 2004; Abrol, 2006). The possibility that cold-hardening processes may operate at behavioural thresholds has received considerably less attention than those at the lethal threshold. However, Mellanby (1939) and Meats (1972) showed effects of rapid acclimation on increasing insect movement at low temperatures, and recent studies have shown that both rearing at low temperature (e.g. 10°C) and rapid cold-hardening can lower the torpor temperature by

several degrees in some species (Kelty and Lee, 2001; Powell and Bale, in press). Shreve *et al.* (2004) showed that rapid cold-hardening increased courtship behaviour and mating at low temperatures in *Drosophila melanogaster*. Such discoveries demonstrate that studies on insect thermal biology could benefit from adopting a wider perspective, in recognition of the limits placed on many insect behaviours by low temperature (Harrison and Roberts, 2000), and the consequent potential for selection for hardening ability at these thresholds (Shreve *et al.*, 2004).

7.3 *Episyrphus balteatus* as a model

The merit of adopting a more holistic view of insect thermal biology is exemplified by the studies on *Episyrphus balteatus* in this project. *E. balteatus* and the closely related *Syrphus ribesii* are two of the most common hoverflies found in the UK during the summer. The larvae are aphidophagous, and are important natural enemies of pest aphids in agricultural and horticultural systems, including *Myzus persicae*, *Sitobion avenae*, and *Aphis fabae*, and much research has been dedicated to understanding this role and determining ways in which agricultural regimes can be adapted to benefit these species (Chambers *et al.*, 1983, 1986; Cowgill *et al.*, 1993; Gilbert, 1993; Tenhumberg and Poehling, 1995). At the end of the summer, the thermal biology of *E. balteatus* and *S. ribesii* follows two distinct routes. The latter species is cold hardy, and the first strongly freeze tolerant insect to be described from the UK, surviving exposure to -25°C (Hart and Bale, 1997a). The necessity of adopting a freeze tolerant strategy has been hypothesised to relate to the dominant overwintering site (wet leaf litter), where the likelihood of external nucleation of freezing is high, and in the absence of freeze tolerance, lethal (Hart and Bale, 1997a).

In contrast, *E. balteatus* does not overwinter in the UK in large numbers, but migrates from southern Europe in the spring, with return movements probable in the autumn (Aubert *et al.*, 1976; Aubert and Goeldlin, 1981; Ball and Morris, 2000). *E. balteatus* is much less cold hardy than *S. ribesii,* and, in terms of its lower lethal threshold, is unable to acclimate: Hart and Bale (1997b) found that survival of *E. balteatus* was only 10% after 10 days at temperatures from 5 to -5°C, and was zero after 10 weeks in UK winter field conditions. It is therefore evident that *E. balteatus* would be unable to overwinter in the UK, although some individuals may survive in thermally buffered environments (Stubbs, pers. comm.), as has been described for the housefly *Musca domestica*, a typical 'opportunistic survivor' (Bale, 1996). The lack of inherent cold tolerance and the inability to seasonally acclimatize may be linked to the necessity for migratory avoidance of northern European winters in *E. balteatus* (Hart and Bale, 1997b). However, even with our current understanding of the lethal effects of low temperature on this species, there are many other aspects of its thermal biology that have apparently not been investigated, and some of these areas have formed the focus for the studies described in this thesis.

7.4 Value of comparing different life stages

Many of the previous studies on rapid cold-hardening have tended to focus on particular stages of the life cycle, especially where one stage had been identified as the one in which the species overwinters, such as the pupa of *S. crassipalpis* (Lee *et al.*, 1987; Chen *et al.*, 1987; Joplin *et al.*, 1990; Chen *et al.*, 1991). The work described in chapter 3 appears to be the first study in which the ability to rapidly cold-harden has been investigated in all stages of an insect's life cycle. Consideration of the differences in cold hardiness between the life stages of a species addresses the differences in function of, in particular, the

resource-acquiring larva and the reproducing adult. This is in addition to the overwintering stage where one is present. Differences in function may require different cold hardiness profiles. For example, such a distinction has been shown in adults of the Monarch butterfly, *Danaus plexippus*, where migratory (post-reproductive) individuals were significantly more cold hardy (lower supercooling point and higher survival of chilling injury) than reproductive individuals (Troyer *et al.*, 1996).

7.5 Relationship between SCP and LLT limits capacity for RCH

Moreover, in the light of the observations that rapid cold-hardening also modifies the SCP in certain polar arthropods (Worland and Convey, 2001; Hawes, pers. comm..), it was of interest to investigate the interactions between rapid cold-hardening and mortality that are attributable to freezing and non-freezing effects. As has been observed in many species, the SCP varied greatly in different stages of the *E. balteatus* life cycle, being lowest in the egg (-25°C) and pupa (-17°C), and highest in the second and third larval instars (-7°C and -6°C respectively). The low SCPs in eggs and pupae have been observed in many insects, as no feeding occurs in these stages (e.g. Coulson and Bale, 1990). These are therefore the stages in which there is the greatest theoretical capacity for rapid cold-hardening, i.e. there is likely to be a measurable difference between the lower lethal temperature and the SCP. The data obtained show that in life cycle stages with a high SCP that is not depressed by acclimation, the freezing event limits rapid cold-hardening depression of the lethal temperature. This was particularly evident in the third larval instar. However, in those stages in which the SCP is much lower than the lethal temperature, there is not necessarily a rapid cold-hardening response, as shown in the egg. It is possible that rapid cold-hardening requires some level of biochemical or physiological organisation that is absent

in *E. balteatus* eggs, or that their low LT_{50} (-19°C) and SCP (-25°C) provides sufficient cold hardiness without the operation of rapid cold-hardening. However, the investigation of rapid cold-hardening in eggs is a new area of inquiry, and the eggs of the locust *Locusta migratoria* do show rapid cold-hardening during slow cooling (Wang and Kang, 2005). The life cycle stages in which the most characteristic rapid cold-hardening was observed were the pupa and first larval instar, both stages with low mean SCPs relative to their lower lethal temperatures. No rapid cold-hardening response was found in adults, again emphasising their limited cold hardiness (Hart and Bale, 1997b).

7.6 Variable RCH capacity at different thresholds is consistent with the ecology of *E. balteatus*

In *E. balteatus* optimal hardening tended to occur during: (1) very short treatments, under 60 minutes in almost all cases; (2) treatment at temperatures above 10°C; and (3) at faster (1.0 and 0.5°C min^{-1}) rather than slower cooling rates. Each of these points is in general contrast to most species in which rapid cold-hardening has been investigated (e.g. *S. crassipalpis* (Lee *et al.*, 1987; Chen *et al.*, 1987; Joplin *et al.*, 1990; Chen *et al.*, 1991)) where optimal hardening usually occurs with longer exposures (2-6 hours), lower temperatures (0-5°C), and slower cooling rates (e.g. 0.1 and 0.05°C min^{-1}).

The generally weak cold-tolerance indicated by these results was further borne out in the experiments on rapid cold-hardening at the threshold for chill coma (chapter 5). Here, hardening had no significant effect on the temperature at which adult *E. balteatus* enter chill coma, in contrast to other species in which this has been investigated (e.g. *Drosophila melanogaster* (Kelty and Lee, 2001) and *Sitobion avenae* (Powell and Bale, in

press)). The coma temperature of adult *E. balteatus* cooled at the slowest rate (0.05°C min^{-1}) was approximately 2°C, and it is likely that this temperature would be encountered in nature, so the lack of hardening is perhaps surprising. However, the results of the experiment on chill-coma recovery time indicated that individuals might harden with respect to knock-down time at low temperatures, so it is not possible to fully conclude that adult *E. balteatus* have no hardening response to chill-coma.

The lack of obvious hardening with respect to lethal and coma-inducing temperatures in adult *E. balteatus* lends weight to the hypothesis that low temperature limits its ability to occur year round in the northern parts of its range, as argued by Hart and Bale (1997b). Thus, given the significance of migration to this species' life history, its low temperature flight threshold is of interest. This study is believed to be the first to investigate the operation of rapid cold-hardening on the flight threshold of an insect, and a clear rapid cold-hardening response was identified (chapter 4). A variety of hardening treatments both increased the initiation of flight at low temperatures, and lowered the overall flight threshold, as measured by an FT_{50} value equivalent in concept to that of LT_{50} or ED_{50} in other areas of research. This method enables characterisation of the hardening process across the relevant threshold, rather than at a single discriminating temperature typical of many studies of rapid cold-hardening (Wang *et al.*, 2003; see also method of Meats, 1973).

As discussed above, this process may be of benefit in enabling flight, and consequently other flight-dependant behaviours, to continue at decreased temperatures. This result is consistent with the hypothesised adaptive significance of the rapid cold-hardening phenomenon as a mechanism of continual fine-tuning of an individual's physiology to its

thermal environment, as described by Meats (1973) and Shreve *et al.* (2004). Relating the 2°C lower flight threshold in hardened flies recorded in this study to a simple analysis of mean monthly maximum temperatures in the Midlands area of the UK (UK Meteorological Office, Sutton Bonnington station data, 1991-2001) suggests that the monthly mean maximum fell below the FT_{50} for non-hardened flies for more months each year than it did for hardened flies (see Table 7.1). These figures illustrate a potential method of linking the action of hardening processes on behavioural thresholds to species' phenology.

Table 7.1 Mean FT_{50} of non-hardened and hardened (treatments pooled) *Episyrphus balteatus* (from data presented in chapter 4), and the mean number of months per year (1991-2001) when mean monthly maximum air temperature was below the FT_{50} (temperature data from Sutton Bonnington recording station, UK Meteorological Office, 2006).

		Mean FT_{50} (°C)	Months per year below FT_{50}
Non-hardened	Male	19.3	10.5
	Female	17.5	9.2
Hardened	Male	16.9	8.7
	Female	15.0	7.6

7.7 Temperature, phenology and climate warming

Understanding of the relationship between temperature and flight in *E. balteatus* was further developed in the analysis of the record of its phenology at a UK field site over a thirty-year period (chapter 6). Strong correlations between temperature and the dates of first and last record, and to a lesser extent annual abundance, were identified, indicating

that temperature is a key determinant in the migratory phenology of *E. balteatus*. Many studies have identified changes in phenology in response to climate warming (Pollard, 1991; Roy and Sparks, 2000; Sparks and Menzel, 2002; Menzel *et al.*, 2006), and analysis of the record of *E. balteatus* showed that the flight period of this species has also become earlier in recent decades. This was the case for not only the date of first record, but also for that of last record. This is in contrast to evidence in the literature for changes in Autumn events, which is generally less strong than that for Spring events, and where it exists tends to indicate events becoming later (Menzel *et al.*, 2006). Although the *E. balteatus* models varied in the predicted number of days by which record dates had advanced for every 1°C increase in temperature, the minimum estimate was 4.6 days, a figure comparable to that described for many UK butterflies (Roy and Sparks, 2000). Climate warming may therefore have significant effects on the phenology of *E. balteatus*. The species is widespread and abundant across its range (Rotheray, 1989; Gilbert, 1993), and the results of this study suggest that flight period is not decreasing, a factor associated with low conservation threat in European hoverflies (Sullivan, 2000). However, effective predation of aphids in cereal crops has been shown to depend on synchronicity between aphid build-up and early oviposition by *E. balteatus* females (Tenhumberg and Poehling, 1995). Significant changes in the phenology of *E. balteatus* could thus have implications for its importance as a natural enemy.

7.8 Sex differences in RCH identified and linked with phenology

In this study, each of the experiments has investigated the possibility of there being different thermal thresholds in males and females, a gender effect that is often omitted or overlooked in investigations of thermal biology (Renault *et al.*, 2002). Sex-specific

aspects of insect thermoregulation include the metabolic rate during running in *Phorocantha* sp. (Rogowitz and Chappell, 2000), and the dependence of flight speed on temperature in *Vespa cabrio* (Spiewok and Schmoltz, 2006). In *E. balteatus*, the daily behaviours of the two sexes, are different: while males remain primarily in 'leks' near aphid colonies, either hovering or resting on foliage, females feed more and forage much more widely (Gilbert, 1984). Sex differences in hardening capacity could thus also provide insight into its functional role, and perhaps underlying mechanisms (Renault *et al.*, 2002). This study has shown that males and females have similar lethal thresholds, and because these are close to the supercooling point, the ability to rapidly cold-harden is limited (chapter 3). There was also no general difference between the chill coma temperature of males and females, nor the time taken to recover from coma (chapter 5). The lack of hardening at this threshold was surprising, as a decrease in CT_{min} through rapid cold-hardening has been shown in other species (Meats, 1973; Kelty and Lee, 2001; Powell and Bale, in press), but this may again be related to the species' generally weak cold hardiness, as demonstrated in survival experiments (chapter 3; also Hart and Bale, 1997b). The lower lethal limits identified (below -6°C) would rarely be encountered by this species. Consequently, it may be argued that the threshold for flight is of greater functional relevance to adult *E. balteatus.* Here, a significant sex difference was identified, the flight threshold being significantly lower (by nearly 2°C) in females than in males, and this difference was maintained in hardened flies, even though the thresholds were reduced compared to non-hardened flies. This gender difference is particularly interesting in comparison with the models of phenology and temperature (chapter 6), where it was shown that the date of first record was both earlier, and showed greater independence of temperature, in females. This result is consistent with females being able

to fly at lower temperatures, and demonstrates the importance of the flight threshold, and consequently the ability to rapidly cold-harden at this threshold, to *E. balteatus*.

7.9 Notes on the methodology of determining behavioural thresholds

Study of thermal thresholds for behaviours has further complications, compared to determining processes at lethal thresholds. In particular, factors additional to physiology affect the expression of those behaviours. For example, flight has clear physiological requirements, including sufficiently high body temperature (Harrison and Roberts, 2000). Insects may fly for a variety of reasons, such as foraging, but the impetus to forage may vary according to circumstances such as how recently an individual has fed, as has been indicated in the beetle *Protostephanus truncatus* (Fadamiro and Wyatt, 1995). Thus while there may well be minimum temperature thresholds below which particular behaviours cannot be expressed, the thermal thresholds below which they are not expressed may vary according to circumstances (Mellanby, 1939). For example, the temperature at which individuals *do* walk under a given set of circumstances, may be very different to that at which they *can* walk if made to do so (Mellanby, 1939). This in itself is interesting, and the relevance of each threshold may be different, but for this reason, caution is advised when drawing ecological conclusions from laboratory measures of thermal behavioural thresholds such as the CT_{min} determined by the glass column method of Huey *et al.* (1992). Methodologies that determine the proclivity, rather than ability, for particular behaviours may be preferable, such as that used here to determine the flight threshold (after Gould, 1998).

7.10 Future research

The need for greater integration of the physiology of cold hardiness with its ecological consequences was recently highlighted by Chown and Storey (2006). This thesis set out to address whether the widely described phenomenon of rapid cold-hardening could be shown to have real implications at ecological thresholds, relating not only to survival of low temperatures, but also to movement (flight and coma). Particular extensions to the work presented could include: (1) the effect of variable hardening temperatures on rapid cold-hardening at the lethal threshold (Chen *et al.*, 1987); (2) the effect of rapid cold-hardening on knock-down time at the chill coma threshold in adults; (3) the operation of rapid cold-hardening at maintaining movement in larvae (Meats, 1973); and (4) the impact on UK phenology of climate in regions from where adult *E. balteatus* migrate to the UK (Sparks *et al.*, 2005).

Low temperature is known to limit movement behaviours such as flight and running (Peng *et al.*, 1992; Harrison and Roberts, 2000; Spiewok and Schmoltz, 2006), and consequently movement-dependant behaviours such as dispersal (Briers *et al.*, 2003) and migration (Campbell and Muir, 2005; Reynolds *et al.*, 2005; Wood *et al.*, 2006), predation rates (Tenhumberg and Poehling, 1995), mate-finding (Svensson and Petersson, 1995) and oviposition behaviour (Ankersmit *et al.*, 1986). Insects may show thermal preferences in microhabitat selection (Hausmann *et al.*, 2005). Temperature also impacts species' phenology (Sparks and Menzel, 2002) and there is evidence for behavioural adaptation of insects to the thermal characteristics of their environment (Hoffmann *et al.*, 2003; Anderson *et al.*, 2005; Dillon and Frazer, 2006).

However, the effect of cold hardening on the maintenance of insect behaviours, and its impact on thermoregulation, is poorly understood. Research in this area has so far focussed on chill coma; most studies have measured the effect of rapid cold-hardening on the critical thermal minimum (Kelty and Lee, 2001; Powell and Bale, in press), though some have used laboratory methods that measure movement proclivity (Meats, 1973), and there has been recent interest in hardening effects on chill coma recovery time (Rako and Hoffmann, 2006).

There is a considerable body of literature on behavioural thermoregulation in insects, including the raising of body temperature by methods such as basking and shivering (Sanborn, 2000), and the dynamics of heat production during flight (Bishop and Armbruster, 1999; Harrison and Roberts, 2002). The work presented in this thesis, in particular the discovery of rapid hardening at the flight threshold (chapter 4), demonstrates that the rapid cold-hardening process has a role in the preservation of behaviours at low temperatures and can be viewed within the context of insect behaviour and thermoregulation. Potential laboratory measures on which the effect of rapid cold-hardening could be investigated could include the low-temperature thresholds for flight and walking (Meats, 1973; Gould, 1998), flight/walking speed or distance using a tethered roundabout or running wheel (Rogowitz and Chappell, 2000; Spiewok and Schmoltz, 2006), mating behaviour (Shreve *et al.*, 2004), the time taken to enter or recover from chill coma (Rako and Hoffman, 2006), and thermal preferences expressed in choice arenas (Hausmann *et al.*, 2005). Such studies should also recognise: (1) the differences between ability and proclivity in the expression of behaviours (Mellanby, 1939); (2) the opportunity to distinguish sex-specific or life-stage-specific behaviours, requirements or hardening responses (Rogowitz and Chappel, 2000; Renault *et al.*, 2002; Shreve *et al.*,

2004); and (3) the complexity of the relationship between ambient temperature and an insect's equilibrium temperature, particularly during high-activity behaviours such as flight and running (Bressin and Willmer, 2000; Harrison and Roberts, 2000). This new interdisciplinary approach may provide one avenue towards greater integration of the study of cold hardening physiology and its ecological and adaptive significance.

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