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RESEARCH ARTICLE

Cross-generation plasticity in cold hardiness is associated with diapause, but not the non-diapause developmental pathway, in the blow fly *Calliphora vicina*

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ABSTRACT

Predicting insect responses to global climate change involves understanding cross-generation effects of temperature. The majority of temperate insects overwinter in a state of diapause, a pre-emptive response to winter conditions associated with increased cold hardiness. Diapause is often induced following maternal adult detection of an environmental cue signifying the onset of winter, whilst diapause is initiated in a subsequent life stage and/or generation. Continued global warming will expose adults to higher late-autumn temperatures, whilst diapause life stages will still experience prolonged winter cold. The cross-generation effect of temperature was investigated by acclimating adult *Calliphora vicina* to present-day (15°C) and future (20°C) late-autumn conditions and assessing cold-hardiness in diapause (D15 and D20) and non-diapause (ND15 and ND20) progeny. A cross-generation plasticity in cold hardiness was associated with D but not ND larvae. D15 larvae exhibited an enhanced ability to suppress internal freezing (supercooling point=–18.9±0.9°C) compared with D20 (−15.3±0.8°C), and displayed a greater tolerance of prolonged exposure to −4°C (LT_{50}=26.0±1.0 and 11.4±1.1 days, respectively) and −8°C (5.1±1.1 and 3.0±1.1 days, respectively). These changes were associated with a reduced glucose content in D15 (2.4±0.3 g mg^{-1}) compared with D20 (3.0±0.3 g mg^{-1}) larvae. In conclusion, *C. vicina* adults exposed to warmer autumn conditions during diapause induction will produce larvae with a reduced cold hardiness capacity, which could negatively impact winter survival. Given that maternal regulation of diapause is common among temperate insects, this could be a widespread phenomenon.

KEY WORDS: Insect, Diapause, Cross generation, Cold hardiness, Climate change

INTRODUCTION

Insects residing within the temperate zone experience yearly winter cycles of low temperatures, potentially freezing conditions and limited nutrient availability. To enhance the chances of survival during this period, insects either migrate to more favourable locations, which offer sufficient resources for growth and reproduction, or remain *in situ*, where physiological and biochemical adaptations are implemented to assist winter survival (Tauber and Tauber, 1976; Danks, 1987; Danks, 2002). The majority of temperate insects utilise the latter approach and enter a dormant state called diapause (Denlinger and Lee, 2010). Diapause is a genetically programmed, pre-emptive response to adverse environmental conditions (Denlinger, 2002), which synchronises active life stages with more favourable conditions and enhances winter survival (Danks, 2002; Hahn and Denlinger, 2007).

Diapause can be either obligatory or facultative (Tauber et al., 1986). Obligatory diapause occurs at a specific stage in the life cycle irrespective of prevailing environmental conditions, while facultative diapause is induced following detection of specific environmental cues (Denlinger, 2002). The dominant induction cue is day length (photoperiod), which has provided a robust indicator of approaching winter conditions throughout evolutionary time (Saunders, 1997; Saunders, 2013). Induction occurs during a sensitive stage in the life cycle, and the critical day length (CDL) denotes a photoperiod that induces 50% diapause within a population (Tauber et al., 1986). The sensitive stage is species specific, and very often at an earlier life stage (or generation) to the one that enters diapause (Tachibana and Numata, 2004; Kostál, 2006; Salminen and Hoikkala, 2013). For example, in the blow fly *Calliphora vicina* (Robineau-Desvoidy 1830), the adult female (sensitive) stage experiences the CDL to induce diapause, whilst diapause initiation does not occur until the third larval instar of the subsequent generation (Saunders, 1987). This maternal regulation of diapause is commonly expressed in temperate insects. Thus, diapause is usually induced well in advance of winter and any exposure to cold stress (Tauber et al., 1986). The CDL typically increases with latitude to account for the earlier onset of winter conditions (Tauber and Tauber, 1972). In the UK, CDLs for *C. vicina* range between 14.5 and 15.5 h (Hayward and Saunders, 1998), which places the timing of diapause induction around early September.

The use of pre-emptive environmental cues to programme diapause provides sufficient time to prepare for the arrival of adverse conditions (Hahn and Denlinger, 2011). This includes the accumulation of energy reserves, reduced metabolic activity and locating an overwintering site (Denlinger, 2002; Koštál, 2006). Once diapause is initiated, there is often an increase in cold hardiness (Goto et al., 2001; Khodayari et al., 2013), associated with the implementation of stress response mechanisms including synthesis of blood sugars, polyols and amino acids (Koštál and Simek, 2000; Koštál et al., 2011a; Koštál et al., 2011b), anti-freeze proteins, heat shock proteins (Rinehart et al., 2007) and alterations to the lipid membrane bilayer (Robert Michaud and Denlinger, 2006). These changes often coincide with an ability to lower the internal freezing point to a temperature known as the supercooling point (SCP) (Zachariassen, 1985; Zachariassen and Husby, 1982; Hahn and Denlinger, 2011), achieved through reduced water content (Zachariassen et al., 2004), removal of ice-nucleating agents (Duman, 1982) and synthesis of blood sugars and ions (Bale, 2002). Whilst it is recognised that the SCP is not an ecologically relevant measure of cold hardiness for the majority of insects (Bale, 1987),

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List of symbols and abbreviations

CDL	critical day length
D	diapause
DT	discriminating treatment
L1	first instar
L3	third instar
LT_{10}	lethal time (days) taken to induce 10% mortality
LT_{50}	lethal time (days) taken to induce 50% mortality
ND	non-diapause
RCH	rapid cold hardening
SCP	supercooling point

it does provide an indicator of physiological changes induced by different temperature conditions.

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

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

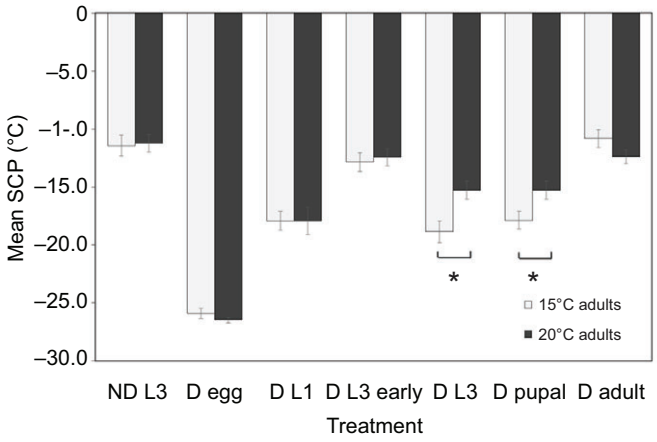

Fig. 1. Mean (±s.e.m.) supercooling point (SCP; °C) of *Calliphora vicina* diapause (D) and non-diapause (ND) progeny at different stages of development from adults acclimated to either 15°C or 20°C. Means marked with an asterisk are significantly different to each other for each developmental stage (*P<0.05, Bonferroni *post hoc* test; N=465).

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

RESULTS

Supercooling capacity

Acclimating adults to either 15°C or 20°C had no influence on the SCP of ND larvae (P=0.87; Fig. 1). For this reason, the effect of adult acclimation on the supercooling capacity of ND progeny was not investigated any further. There was an effect of adult acclimation on the SCP of D larvae, with D15 larvae (SCP=–18.9±0.9°C) exhibiting significantly lower SCPs than D20 larvae (SCP=–15.3±0.8°C, P<0.05; Fig. 1). A similar response was detected in pupae from 15°C adults (P<0.05; Fig. 1). No response to adult acclimation was detected at any other life stage for D programmed progeny. D larvae from both adult temperature regimes had significantly lower SCPs than ND larvae (P<0.001).

There were significant differences in the SCPs of D larvae produced from the cross-fertilisation of parental males and females held at either 15°C or 20°C ($F_{4,95}$=8.0, P<0.001; Fig. 2). The SCP was more strongly influenced by maternal than paternal acclimation temperature, with crosses involving females held at 15°C having the lowest mean SCPs (♂20 × ♀15=–16.0±0.7°C; ♂15 × ♀15= –19.3±0.5°C). *Post hoc* analysis found the SCPs of D larvae from ♂15 × ♀15 to be significantly lower than D larvae from ♂20 × ♀15 (P<0.05, Bonferroni *post hoc* test) and ♂15 × ♀20 (P<0.001). The difference between the ♂15 × ♀15 cross and the ♂20 × ♀20 cross was very close to significant (P=0.07) and was shown to be significant if using the less conservative LSD *post hoc* test (P<0.05).

Lethal times

Adult acclimation did not affect the cold hardiness of ND larvae, with the exception of lethal time (days) taken to induce 10% mortality (LT_{10}) at −4°C (Fig. 3).

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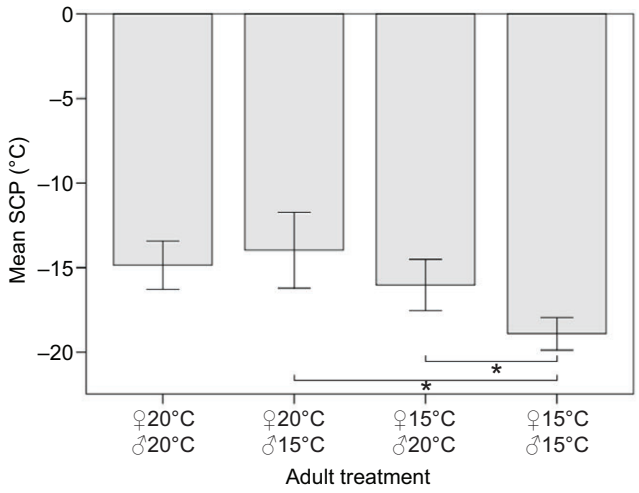

Fig. 2. Mean (±s.e.m.) SCP (°C) for *C. vicina* D progeny from different crosses between adult males and females separated at eclosion and acclimated to 15°C or 20°C. Males and females were combined/allowed to mate after 10 days of acclimation, and continued to be held at the temperature experienced by the female. Means marked with an asterisk are significantly different to each other for each developmental stage (*P<0.05, Bonferroni *post hoc* test; N=95).

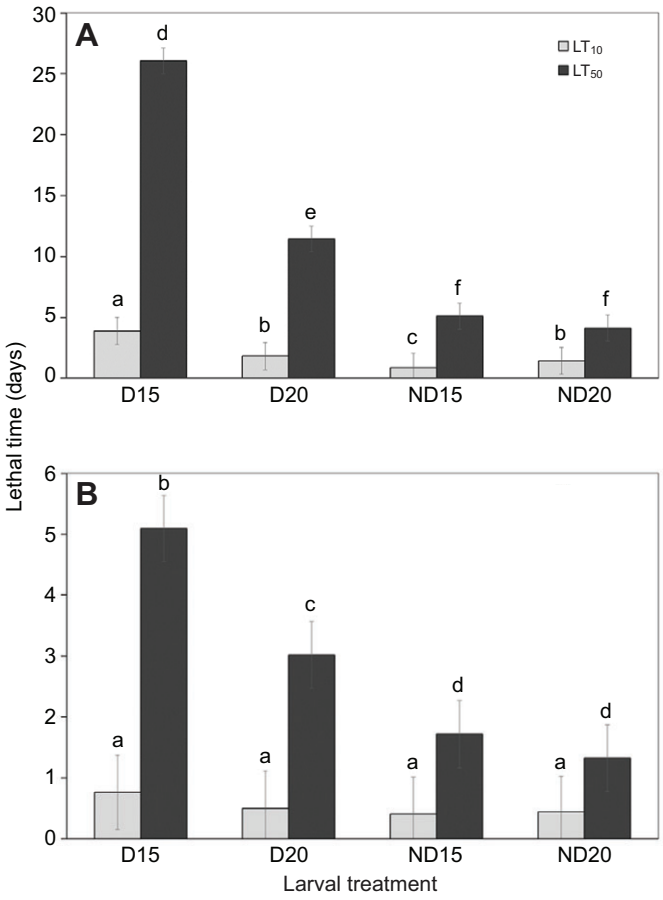

Fig. 3. The mean lethal time (±s.e.m.) (days) survival for *C. vicina* D and ND larvae from adults acclimated to 15°C (ND15, D15) and 20°C (ND20, D20). Lethal time was determined as the time taken to induce 10% (LT_{10}) and 50% (LT_{50}) mortality following exposure to (A) –4°C and (B) –8°C. Means marked with different letters are significantly different, as determined by non-overlapping fiducial limits.

D larvae were significantly more cold tolerant than ND larvae, as determined by non-overlapping fiducial limits of LT values (Fig. 3). D15 larvae were also significantly more cold tolerant than D20 larvae. For D larvae this was detected following all treatments, with the exception of LT_{10} at −8°C (Fig. 3B), and was most evident at −4°C, with a 14.6 day difference in the LT_{50} (lethal time taken to induce 50% mortality) value between D15 (26.0±1.0 days) and D20 larvae (11.4±1.1 days).

Rapid cold hardening

The discriminating treatments (DTs; see Materials and methods) of ND and D larvae were not significantly influenced by adult temperature ($F_{4,24}$=0.35, P=0.71; Fig. 4). The DT for ND15 and ND20 was slightly higher (2 h at −10°C) than for D15 and D20 larvae (2 h at −11°C), although this difference was not significant (P=0.71). DT survival ranged from 21.7±1.7% for ND15 and ND20 larvae to 26.7±3.3% for D15 larvae.

A pre-treatment of 2 h at 0°C prior to DT exposure was associated with a significant increase in survival relative to direct transfer to DT for ND20 (survival 45±4.2%; P<0.001), ND15 (survival 35±2.2%; P<0.001) and D20 larvae (survival 45±4.3%; P<0.05), but not for D15 larvae (survival 35±2.2%; P=0.35; Fig. 4). The effect of parental temperature was not significant for either D (P=0.065) or ND larvae (P=0.065).

Gradual cooling at a rate of 0.5°C min^{-1} to the DT induced a stronger RCH response than the constant temperature pre-treatment, and survival of all larvae was significantly greater than for their respective DT (P<0.001 for all). There was also a significant difference in survival between ND and D larvae ($F_{4,24}$=8.5, P<0.001). However, *post hoc* analysis did not identify a significant difference in survival associated with adult acclimation temperature for either D (P=0.10) or ND larvae (P=0.26).

Larval mass, water and glucose content

In general, wet mass was lowest for D larvae (Table 1), and significant differences were detected across the four larval treatment groups ($F_{4,80}$=4.9, P<0.01). ND15 larvae had the greatest wet mass, and *post hoc* analysis revealed this was significantly different from that of D15 and D20 larvae (P<0.05 for both). Wet mass was not influenced by adult acclimation for either ND or D larvae (P=1.0 for both). Dry mass was significantly different across all treatments ($F_{4,80}$=4.1, P<0.01), with *post hoc* analysis determining dry mass of ND15 larvae to be significantly greater than that of D20 larvae (P<0.05). Again, this was not influenced by adult acclimation for either ND or D larvae (P=1.0 for both). Water content did not vary between any larval treatment groups ($F_{4,80}$=2.6, P=0.6).

Glucose content was significantly different across treatment groups ($F_{4,20}$=28.7, P<0.001); however, this was not influenced by adult culturing temperature for either D (P=0.3) or ND larvae (P=1.0). Overall, D larvae had significantly higher glucose contents than ND larvae (P<0.001 for all ND versus D comparisons).

DISCUSSION

Cross-generation responses to environmental stressors are well documented in a broad range of organisms (Marshall, 2008; Castro et al., 2013; Plautz et al., 2013; Suter and Widmer, 2013). Understanding these responses will be critical in determining how different organisms will cope with future climate change (Mondor et al., 2004; Rando and Verstrepen, 2007; Burgess and Marshall, 2011; Salinas and Munch, 2012). The findings of this study indicate that elevated temperatures experienced by a parental population of

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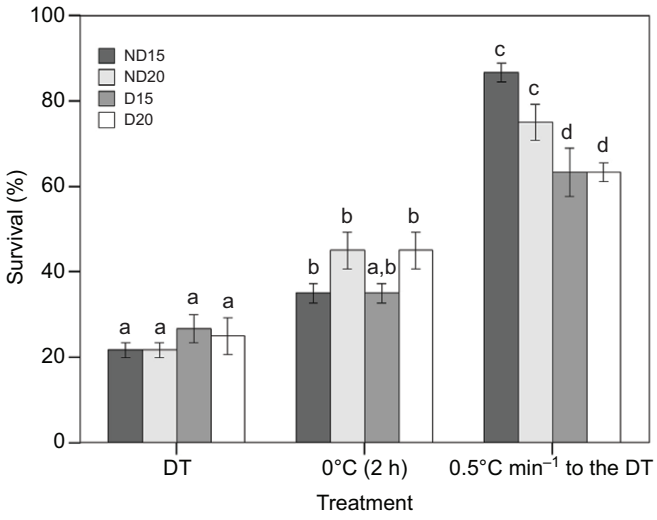

Fig. 4. Survival (%) of non-diapause (ND) and diapause (D) *C. vicina* third instar larvae exposed directly to the discriminating treatment (DT), or following a rapid cold hardening (RCH) treatment of either 2 h at 0°C or gradual cooling at 0.5°C min^{-1}. Larvae from adults acclimated to 15°C (ND15, D15) and 20°C (ND20, D20). Survival was assessed as successful adult eclosion at 11°C. Means ± s.e.m. are presented (N=10×6 replicates per data point). Means marked with different letters are significantly different to each other at P<0.05 (Bonferroni *post hoc* test).

the blow fly *C. vicina*, in autumn or early winter, have the potential to significantly reduce the cold hardiness of overwintering D larvae in the subsequent generation. Given the widespread occurrence of a maternally regulated diapause (Mousseau and Dingle, 1991), this could be a prevalent phenomenon for insects overwintering in the temperate zone.

The cross-generational influence of adult thermal history on larval cold tolerance (as determined by SCPs and LT_{50}) was not seen within the non-diapause developmental pathway, but only as part of diapause. Even within the developmental pathway of diapause-programmed flies, the SCP of eggs, first instar (L1) and even early stage third instar (L3) larvae were not affected by adult temperature. This only occurred in the L3 larval stage, i.e. after the start of diapause (Fig. 1). Consequently, this study further reinforces a direct physiological relationship between both the programming and initiation of diapause and increased cold hardiness. Increased cold tolerance has been associated with diapause in a number of species (Kostál and Simek, 2000; Goto et al., 2001; Vesala and Hoikkala, 2011) and some of the underlying cold tolerance mechanisms are well understood (Arrese and Soulages, 2010; Denlinger and Lee, 2010; Khodayari et al., 2013). However, the mechanisms linking the photoperiodic programming of diapause with variation in temperature, and the subsequent cross-generational control over cold tolerance phenotypes in the next generation, remain a black box. The evidence from male × female crosses held at different temperatures (Fig. 2) indicates that it is the thermal history of the mother that has the greatest influence over diapause-associated cold tolerance. This is in agreement with a previous study in *C. vicina* where both diapause incidence and duration phenotypes were shown to be mainly controlled by the maternal line (McWatters and Saunders, 1998). This phenomenon has also been identified in other species, for example the mosquito *Aedes togoi* (Kappus and Venard, 1967) and the parasitic wasp *Trichogramma evanescens* (Vinogradova and Zinovjeva, 1972). It is important to note, however, that diapause incidence was 100% in all D15 and D20 samples used in the present study, i.e. all larvae were selected after 30 days post-oviposition, which denotes the start of diapause in this species (Richard and Saunders, 1987). Thus, the cold hardiness differences observed are not a consequence of differences in diapause incidence. Instead, there would appear to be a separate cross-generational mechanism controlling plasticity in the cold tolerance phenotype of diapausing larvae that is distinct from the physiological 'decision' to enter diapause. To our knowledge, this is the first evidence of such a trans-generational effect on cold hardiness in insects, and clearly warrants further investigation.

Diapausing *C. vicina* exhibited a stronger supercooling capacity compared with ND larvae irrespective of parental temperature conditions (Fig. 1), and the lowest SCPs were associated with a lower parental acclimation temperature. It is important to recognise that SCP temperatures represent the physiological limit of supercooling, and not the ecological limit of survival (Bale, 1996; Renault et al., 2002). However, a number of studies have identified a correlation between supercooling capacity and low temperature tolerance, including in the heteropteran *Pyrrhocoris apterus* (Hodkova and Hodek, 1997) and the coleopteran species *Coccinella septempunctata* and *Semiadalia undecimnotata* (Kalushkov and Nedved, 2000). For this reason, the SCP continues to be used to provide insight in to the physiology of cold hardiness and as a useful comparative index (Renault et al., 2002). As found in a previous study (Hayward and Saunders, 1998), D larvae were also significantly more cold tolerant than ND larvae (Fig. 3). Parental thermal history only had an effect within the diapause programme, and differences in cold tolerance were greatest between D15 and D20 larvae following exposure to −4°C, with a 15 day difference in LT_{50} values. The causes of mortality at sub-zero temperatures include cellular damage, enzyme blockage and the accumulation of metabolites which become toxic at high concentrations (Renault et al., 2002). Protective mechanisms against these damaging effects are a well-known part of diapause (Hahn and Denlinger, 2007; Rinehart et al., 2007; Rozsypal et al., 2013; Teets and Denlinger, 2013), and our study suggests that the cross-generational programming of at least some of these stress tolerance mechanisms are influenced by the thermal, and not just photoperiodic, history of the parental generation.

Despite these differences in chronic cold tolerance, there were no differences in survival following acute cold stress between D and

Table 1. Mean (±s.e.m.) wet mass, dry mass, water content, glucose content for non-diapause (ND) and diapause (D) *C. vicina* larvae from adults acclimated to 15°C (ND15, D15) and 20°C (ND20, D20)

	ND15	ND20	D15	D20
Wet mass (mg)	83.0±2.0^{a} (65.0–97.0; n=20)	83.2±1.9^{b} (69.0–96.0; n=20)	74.9±1.4^{a,b} (63.0–86.0; n=20)	78.4±2.0 (56.0–89.0; n=20)
Dry mass (mg)	25.2±0.4^{c} (22.0–29.0; n=20)	24.9±0.5 (21.0–30.0; n=20)	24.1±0.4 (21.0–27.0; n=20)	23.4±0.4^{c} (21.0–26.0; n=20)
Water content (% of dry mass)	69.5±0.7 (60.8–72.3; n=20)	69.9±0.5 (65.7–76.0; n=20)	67.7±0.6 (61.9–73.8; n=20)	69.9±0.8 (62.5–76.0; n=20)
Glucose content (g mg^{-1})	0.9±0.2^{e} (0.4–1.3; n=5)	0.6±0.2^{f} (0.1–1.1; n=5)	2.4±0.2^{e,f} (1.8–2.6; n=5)	3.0±0.3^{e,f} (2.0–3.6; n=5)

Ranges and sample sizes are given in brackets. Means followed by the same letter are significantly different at P>0.05 (Bonferroni *post hoc* test).

ND larvae, as determined by the DT (Fig. 4). Both ND and D larvae demonstrated RCH, with a slight increase in survival following 2 h at 0°C, and a significant increase in survival following gradual cooling to DT at 0.5°C min^{-1} (Fig. 4). The strongest RCH response was seen in ND larvae, which supports the idea of diapause being an anticipatory response to chronic winter cold (Tauber et al., 1986) rather than an immediate response to extreme and acute cold shock. For diapausing *C. vicina* in particular, the winter period is spent in the thermally buffered soil microhabitat (Hayward and Saunders, 1998), where selective pressures to enhance tolerance to prolonged cold exposure are stronger than evolutionary pressure to tolerate sudden temperature fluctuations (Saunders, 1987; Vaz Nunes and Saunders, 1989). Adult acclimation had no effect on the RCH ability of D or ND larvae following 2 h exposure to 0°C prior to DT exposure, or gradual cooling to the DT. However, ND larvae from 15°C adults showed a markedly stronger RCH response (86.7±2.1%) than ND larvae from 20°C parents (75.0±4.3%; Fig. 4). While this difference was not significant, it may warrant further investigation, and would represent a cross-generation control of cold tolerance that was not linked to the diapause programme.

Previous work on *C. vicina* has identified glucose as the dominant compound in all ND life stages (Block et al., 1990). However, whether levels of glucose change as part of the diapause programme has never been investigated in this species. Increased levels of glucose have been strongly associated with diapause in several other insects (Pullin and Wolda, 1993; Overgaard et al., 2007; Robert Michaud et al., 2008; Hou et al., 2009; Ragland et al., 2010; Xu et al., 2012), with a hypothesised role in in enhancing cold tolerance. We noted a threefold increase in glucose concentration in *C. vicina* D larvae (Table 2), which, combined with greater cold tolerance (Fig. 3) and lower SCPs (Fig. 2), provides further correlative evidence for glucose potentially contributing to increased cold hardiness. However, we also noted a slight reduction (though not significant) in glucose levels between D20 and D15 larvae, despite the latter being significantly more cold tolerant and having a lower SCP. Thus, it seems that glucose may not contribute directly to cold tolerance in this instance, but could instead be being metabolised into other cryoprotectants in D15 larvae, such as trehalose and glycogen, both of which are associated with enhanced cold tolerance (Storey, 1997; Arrese and Soulages, 2010). The specific role of glucose clearly requires further investigation, although it has been associated with both diapause and RCH in previous studies (Robert Michaud and Denlinger, 2007; Overgaard et al., 2007; Robert Michaud et al., 2008; Hou et al., 2009). However, it is unclear whether upregulation is due to a role in cold protection or is a by-product of glycolysis (Robert Michaud and Denlinger, 2007).

Phenotypic plasticity has been described as a bet-hedging strategy to enhance survival in an unpredictable environment (Simons and Johnston, 1997). Both costs and benefits are associated with acclimation (Huey et al., 1999), although the species most at threat from climate change are those in which evolutionary and plastic adjustments are either impossible or slower than changing environments (Chown et al., 2010). The phenotypic adjustment identified here would prove beneficial if higher adult temperatures in autumn were indicative of warmer conditions for larval progeny during winter (as suggested by the beneficial acclimation hypothesis) (Wilson and Franklin, 2002). However, climate models predict that while autumns will continue to get warmer (IPCC, 2007), the winter will continue to experience periods of prolonged cold. A recent study even suggests that as Arctic ice sheets continue to melt, winters will become longer and colder across Europe and North America (Jaiser et al., 2012). Here we have presented a previously unrecognised threat, that parental thermal history has a cross-generational effect on the cold tolerance of their diapausing progeny.

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

MATERIALS AND METHODS

The study species

Calliphora vicina used in this study were sourced from the University of Birmingham campus, UK (52.4°N, 1.9°W), in 2009 using olfactory traps (Hwang and Turner, 2005). Since this time, stock cultures have been maintained as outlined by Hayward and Saunders (Hayward and Saunders, 1998) and regularly replenished with wild-caught individuals. Adult cultures for experimental use were established as follows: long-day photoperiod (18 h:6 h light:dark) at 15°C or 20°C to produce non-diapause (ND) larvae (abbreviated to ND15 and ND20, respectively); and short-day photoperiod (12 h:12 h light:dark) at 15°C or 20°C to produce diapause (D) larvae (abbreviated to D15 and D20, respectively).

Diapause larvae were also produced from cross-temperature cultures established under short-day photoperiods by separating males and females at the time of eclosion and culturing them at either 15°C or 20°C. Males and females were combined after 10 days to create the four crosses presented in Table 2, all under short-day photoperiods.

For all cultures, liver was provided as a protein source and site of egg oviposition on days 4, 6 and 8 and every day thereafter. From day 12 onwards, eggs were removed from parental conditions within 18 h of oviposition and transferred to complete darkness under high humidity at 11°C to induce egg hatching (Davies, 1950). Removal of eggs within 18 h

Table 2. Adult temperature treatments used to determine the effect of paternal and maternal thermal history on the supercooling point of *Calliphora vicina* larval progeny

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

All adults were separated according to sex at eclosion, cultured under a short day photoperiod (12 h:12 h light:dark) and crossed after 10 days.

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ensured a synchronous cohort of larvae that all experienced identical conditions. Thus, any subsequent variation in cold hardiness was a response to adult acclimation temperature. D and ND larvae are morphologically indistinguishable; however, D larvae can be identified as larvae not pupariating by day 30 post-oviposition (Saunders, 1987), as the larval ring gland becomes refractory to stimulation by the brain neuropeptide, prothoracicotropic hormone (Richard and Saunders, 1987). For this reason, ND larvae were collected day 15 post-oviposition, while D larvae were identified by a delayed pupariation (Saunders, 1987) and collected day 30 post-oviposition. Thus, diapause incidence in all D samples used was 100%. Unless stated otherwise, ND and D larvae were used for experimental use on days 15 and 30, respectively.

Supercooling capacity

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

Lethal time

The lethal time was determined for ND15, ND20, D15 and D20 larvae. Three groups of 10 L3 larvae were placed in 10 ml glass vials containing 1 cm of sawdust, held in a programmable incubator at either −4°C or −8°C, and removed at 3 day intervals for the first 18 days and then 3 or 6 day intervals until 50 days. Preliminary experimentation found 50 days was necessary to ensure complete mortality. Vials were removed and held at 11°C in darkness for 24 h then at 20°C until eclosion. Larvae failing to eclose after 21 days were considered dead. Controls were maintained at 11°C for the duration of each experiment. A probit analysis was then used to estimate the time taken to kill 10% (LT_{10}) and 50% (LT_{50}) of the population. Methods used to investigate the lethal time are based on previous approaches (Hart et al., 2002; Hughes et al., 2009; Hughes et al., 2010; Hayward and Saunders, 1998). *N*=approximately 120 per treatment.

Rapid cold hardening

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

RCH ability was then determined by holding larvae for 2 h at 0°C before transfer to the DT, and cooling larvae from 11°C to the DT at a rate of 0.5°C min^{-1}. This was determined for ND15, ND20, D15 and D20 larvae (*N*=60 per treatment). Controls were held in 50 ml test tubes at 11°C for the duration of each experiment.

Larval mass, water and glucose content

Following measurement of whole-body wet mass to the nearest milligramme, ND15, ND20, D15 and D20 larvae were dried for 2 days at 70°C to constant mass. Dry mass was then recorded and water content was calculated gravimetrically as a percentage of dry mass. Whole-body glucose content was determined using a Glucose (HK) Assay Kit (Sigma GAHK-20, Sigma-Aldrich, Gillingham, Dorset, UK). Samples of five L3 larvae from the four adult treatments were homogenised and diluted with deionised water. Glucose content was determined spectrophotometrically by measuring absorbance of light at 340 nm. *N*=80 for mass and water content; *N*=20 for glucose content.

Statistical analysis

Analysis of SCPs, RCH, mass, water content and glucose content data was undertaken using SPSS (v. 20.0, IBM, Armonk, NY, USA). Data were first subjected to a Kolmogorov–Smirnov test to identify the distribution that best described results. RCH data were analysed using a multivariate two-way ANOVA whilst all other data were analysed using a univariate ANOVA. Significant differences between data points were identified using the Bonferroni *post hoc* test with significance identified at $P<0.05$.

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

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Competing interests

The authors declare no competing financial interests.

Author contributions

The concept and design of the experiment is the work of P.C.C. and S.A.L.H. The experiment was executed and results were interpreted by P.C.C. Drafting of the article was undertaken by P.C.C., S.A.L.H. and J.S.B.

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