23 The Rapid Cold-hardening Response in Insects: Ecological Significance and Physiological Mechanisms

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Introduction

Historically, investigations of insect cold hardiness have focused on seasonal adaptations acquired over a period of weeks to months, which promote low temperature and winter survival (Zachariassen, 1985; Duman *et al.*, 1991; Lee, 1991; Storey and Storey, 1996). However, in addition to increased winter cold hardiness, many species of insects also possess the ability to quickly, on the order of minutes to hours, increase their cold tolerance at other times of the year. It has now been more than 20 years since the first report of this rapid cold hardening (RCH) response in insects (Chen *et al.*, 1987; Lee *et al.*, 1987). The RCH response is characterized by increased protection against cold shock (i.e. non-freezing) injury following a brief acclimation to moderately low temperature. Lee *et al.* (1987) originally hypothesized that RCH allows insects to 'instantaneously' enhance their cold tolerance by tracking changes in environmental temperature. Since then, strong support for this hypothesis has come from investigations documenting RCH under ecologically relevant cooling regimes and diurnal thermoperiods, and a significant body of literature has demonstrated the broader ecological implications – improved courtship behaviour, mating success and fecundity – of the RCH response. Therefore, the significance of RCH is not restricted to the increased survival of low temperatures, but appears to function more generally to enhance organismal performance within a thermally variable environment.

For many years the mechanisms underlying RCH remained poorly understood and only recently we have begun to gain a better understanding of the physiological mechanisms of the response. However, our knowledge remains limited as to the molecular and cellular events involved in the signalling pathway for induction of the cold-hardening response. The purpose of the present chapter is to review briefly our understanding of RCH in insects, especially as it relates to the ecological implications and physiological mechanisms of the response.

Ecological Significance of Rapid Cold Hardening

Rapid cold hardening increases survival of low temperature

Rapid cold hardening, as its name implies, contrasts with the slow, seasonal attainment of increased cold tolerance associated with preparation for winter. Within minutes to hours, the RCH response can dramatically increase an

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insect's tolerance of cold shock. For example, flesh flies, *Sarcophaga crassipalpis*, reared at 25°C cannot survive direct exposure to −10°C for 2 h (Lee *et al.*, 1987). However, if this low temperature treatment is preceded by RCH at 0°C for 2 h flies readily survive; even as little as 10 min at 0°C allows ~50% of flies to survive subsequent exposure to −10°C. Similarly, in adult fruit flies, *Drosophila melanogaster*, chilling at 5°C for 30 min dramatically increases (from 0 to 50%) survival of subsequent exposure to −5°C for 2 h (Czajka and Lee, 1990). In nature, this response may be especially important to rapidly enhance the cold tolerance of insects during the early spring and late autumn months that are often accompanied by dramatic cold snaps.

Most investigations have induced the RCH response by exposing insects to temperatures between 0 and 10°C for a few hours. However, the acquired cold tolerance is lost rapidly (within ~2 h) if flies are returned to their rearing temperature of 25°C (Chen *et al.*, 1991). Similar response profiles have been reported in most other insects in which RCH has been examined.

RCH appears to be an extremely widespread response among freeze-intolerant insects, having been documented in nearly 30 species, including members of the orders Coleoptera (beetles), Diptera (flies), Hemiptera (bugs and aphids), Lepidoptera (butterflies and moths), Orthoptera (grasshoppers and crickets) and Thysanoptera (thrips). Within a species, the response can occur in multiple developmental stages, including eggs, larvae, pupae and adults (Czajka and Lee, 1990; Wang and Kang, 2005), and among both non diapausing and diapausing individuals (Chen *et al.*, 1987). Recently, we demonstrated that the RCH response not only confers increased tolerance of cold shock, but can also increase freeze tolerance, as we observed in the Antarctic midge, *Belgica antarctica* (Lee *et al.*, 2006b).

Rapid cold hardening during ecologically relevant thermocycles

Most early investigations of RCH induced the response by direct transfer of insects from their laboratory rearing temperature to the cold hardening temperature (e.g. direct transfer from 23 to 0°C for 2 h). While such direct transfers clearly increase subsequent cold tolerance and are useful for exploring the physiological basis of the protection generated, the ecological relevance of such protocols for induction of RCH is questionable. In nature, insect populations rarely, if ever, experience such dramatic fluctuations and extremes of low temperature.

A growing body of literature now indicates that RCH is also induced under ecologically relevant cooling regimes and natural diurnal thermocycles (Coulson and Bale, 1990; Kelty and Lee, 1999, 2001; Koveos, 2001; Powell and Bale, 2004, 2006; Kelty, 2007). For example, an RCH response is induced in *D. melanogaster* during cooling from 23 to 0°C at natural rates (0.05 or 0.1°C/min) (Kelty and Lee, 1999). Further, a significant decrease in the lower lethal temperature is observed when flies are cooled from 23°C to only 11°C, a temperature commonly experienced in nature. Kelty and Lee (2001) demonstrated that the cold tolerance of *D. melanogaster* increased during the cooling phase of an ecologically relevant, diurnal thermocycle and further increased with the number of thermoperiods to which flies were exposed. More recently, RCH has been documented in flies maintained in a field setting and exposed to ambient thermocycles (Koveos, 2001; Kelty, 2007). Such studies clearly demonstrate an ecological relevance for the RCH response and support the notion that RCH allows insects to 'instantaneously' enhance their cold tolerance within a thermally variable environment.

However, most insects will never benefit from the reduction of the lower lethal temperature associated with RCH, as they are unlikely to experience temperatures low enough to cause mortality as a result of direct chilling injury. In this case, for the RCH response to have ecological relevance, it must prevent more subtle deleterious effects of chilling at temperatures that are likely to be encountered within an insect's natural environment. Evidence for this may be seen in the effect of RCH on the critical thermal minimum (CT_{min}), the temperature at which an insect can no longer maintain activity

and enters a cold-induced torpor or chill coma. In *D. melanogaster*, RCH during slow cooling (at 0.1°C/min) lowers the CT_{min} by >2.5°C (to 3.9°C) (Kelty and Lee, 1999). Powell and Bale (2006) reported a similar lowering of the CT_{min} (by ~2.5°C) in the grain aphid, *Sitobian avenae*, during RCH. Further, the CT_{min} of *D. melanogaster* decreased during the cooling phase of a natural diurnal thermocycle (Kelty and Lee, 2001), seemingly 'tracking' changes in ambient temperature. Such reductions of CT_{min} may be explained by the preservation of the resting membrane potential in muscle fibres and nervous tissue, neural conduction velocities and neuromuscular coordination at lower temperatures following RCH (Kelty *et al.*, 1996). Even a relatively modest 1–2°C reduction of the CT_{min} following RCH would allow insects to expand the thermal range over which activity, feeding and reproduction may be maintained, thereby optimizing performance at temperatures lower than otherwise possible.

Rapid cold hardening preserves courtship behaviour, reproductive success and longevity

RCH appears to not only improve survival of low temperatures, but also ameliorates the sublethal effects associated with cold-shock injury (Rinehart *et al.*, 2000; Powell and Bale, 2004; Shreve *et al.*, 2004; but see Coulson and Bale, 1992). For example, in *S. crassipalpis* cold shock at −10°C for 1 h negatively affects adult longevity, egg production and fertilization success (Rinehart *et al.*, 2000). However, RCH for 2 h at 0°C eliminates these negative effects following subsequent low-temperature exposure. Similar benefits of RCH on development, longevity and reproductive success in the aphid, *S. avenae*, were observed by Powell and Bale (2004). In adult *D. melanogaster*, a reduction in temperature from 23 to 16°C resulted in only half (11/22) of male-female pairs engaging in courtship behaviours and no pairs were observed mating (Shreve *et al.*, 2004). However, if flies were allowed to acclimate for 2 h at 16°C, nearly all pairs (17/20) displayed courtship behaviours and more than half (11/20) mated successfully. Further, the hardening response observed by Shreve *et al.* (2004) after 2 h at 16°C is the highest reported induction temperature for an RCH response. These results provide yet further evidence that RCH not only allows rapid enhancement of an insect's cold tolerance, but represents a constant fine-tuning of behavioural and physiological processes to match environmental conditions. Consequently, the term 'rapid cold hardening', with its emphasis on low-temperature tolerance, is in some regards a misnomer, as it is too restrictive to encompass the wide range of induction temperatures and organismal benefits from this swift acclimatory response (Fig. 23.1).

Induction and Physiological Mechanisms of the Rapid Cold-hardening Response

While a relatively clear picture has emerged for the ecological significance of the RCH response, understanding the underlying physiological mechanisms has proved a far greater challenge. This is especially true in light of documented species-specific differences in the biochemical/physiological responses that occur

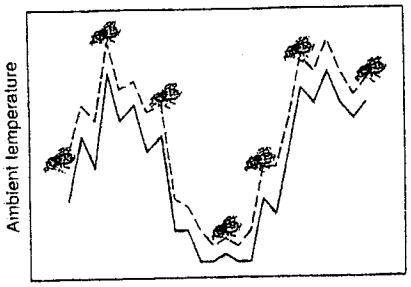

Fig. 23.1. Theoretical representation of the rapid cold-hardening response. Rapid cold hardening allows insects (– – –) to track changes in ambient temperature (——), thereby optimizing their behavioural and physiological performance. Such fine-tuning may be crucial for important fitness traits, such as survival, activity, feeding and reproduction, within a thermally variable environment.

during RCH. More recent investigations have, however, begun to provide deeper insight into the mechanistic basis for the response.

The role of cryoprotectants?

Early investigations as to the mechanisms underlying the RCH response reported a correlation between the accumulation of cryoprotective compounds and the onset of increased cold hardiness (Chen *et al.*, 1987; Lee *et al.*, 1987). For example, cold-hardening pharate adults of *S. crassipalpis* at 0°C for 2 h results in a threefold increase in glycerol levels to ~80 mM (Lee *et al.*, 1987). While modest in comparison to the seasonal accumulations in many overwintering insects, such increases in cryoprotectants likely play a non-colligative role in stabilizing proteins and membrane structures at low temperatures (Carpenter and Crowe, 1988; Crowe *et al.*, 1988). However, later investigations found no evidence for the accumulation of glycerol or other potential cryoprotectants during RCH in *D. melanogaster* (Kelty and Lee, 1999, 2001). Together these results suggest that, at least in some insect species, the accumulation of cryoprotective compounds alone cannot account for the increase in cold hardiness that occurs during RCH.

More recent studies have taken a metabolomics approach to simultaneously monitor the relative concentrations of a large number of metabolites in insects exposed to low temperature and/or RCH. In *S. crassipalpis*, metabolomic analysis confirmed previous reports of the accumulation of glycerol during RCH, but also revealed increased concentrations of a variety of other known cryoprotectants including sorbitol, glucose and alanine (Michaud and Denlinger, 2007). Similarly, using metabolic profiling, Overgaard *et al.* (2007) reported increased concentrations of glucose and trehalose (~1.5- to 2-fold) in adults of *D. melanogaster* during an ecologically relevant bout of cooling that induced RCH. This result differs from previous reports that found no evidence of cryoprotectant accumulation in *D. melanogaster* during RCH (Kelty and Lee, 1999, 2001). Clearly, further study concerning the accumulation and mechanistic role played by cryoprotective compounds in RCH is needed.

Heat-shock proteins and gene expression

The synthesis of heat shock proteins (i.e. stress proteins) and other molecular chaperones is a well-documented rapid response to high temperature exposure in both plants and animals, including insects (Feder and Hofmann, 1999). However, the response of heat-shock proteins in insects exposed to low temperature is less well studied (Denlinger and Lee, 1998). Stress-protein synthesis in response to cold shock has been documented in *D. melanogaster* (Burton *et al.*, 1988; Sejerkilde *et al.*, 2003), *S. crassipalpis* (Joplin *et al.*, 1990) and several other insect species, but appears to occur only during recovery from and not during the low-temperature exposure. For example, when *Drosophila triauraria* were subjected to 0°C for 24 h, *Hsp70* mRNA was not detected immediately after the exposure but accumulated within 30 min upon return to 23°C (Goto and Kimura, 1998). Similarly, exposure of *D. melanogaster* to 0°C for 2 h, a temperature treatment known to induce a significant RCH response, fails to result in the accumulation of *Hsp70* transcripts (Sinclair *et al.*, 2007) or protein (Nielsen *et al.*, 2005; Overgaard *et al.*, 2005). This is consistent with the lack of Hsp70 induction during RCH of *D. melanogaster* using an ecologically relevant, diurnal thermocycle (Kelty and Lee, 2001). Further, Yi *et al.* (2007) found that RCH did not induce a significant increase in the expression of Hsp110, Hsp70 and Hsp27, or the heat-shock factor, Hsf-1. Together, these data suggest that increased expression of heat-shock proteins plays little to no significant role during RCH in insects, but are likely important during recovery from chilling injury. This suggestion is further supported by the finding that *D. melanogaster* undergo RCH even when protein synthesis is inhibited by treatment with cycloheximide (Misener *et al.*, 2001).

A recent investigation using microarray analysis revealed a change in the expression of 37 genes from *D. melanogaster* chilled at 0°C for 2 h followed by a 30 min recovery at 25°C

(Qin *et al.*, 2005). Of the 31 transcripts up-regulated during cold hardening, nearly a third appeared to encode membrane proteins. These findings were perhaps not surprising, as cellular membranes are believed to be the primary site for cold-shock damage (Drobnis *et al.*, 1993; Hazel, 1995) and reorganization of the membrane appears to be involved in the RCH response (see below). A number of other transcripts encoding stress proteins (*Hsp83*, *Hsp26*, *Hsp23*) were also up-regulated and likely function during recovery following chilling and/or in blocking cold-induced apoptosis (Yi *et al.*, 2007).

To date, studies investigating the role of heat-shock proteins in the RCH response have focused solely on single low-temperature exposures and the subsequent recovery from these events. However, in nature, most insects are exposed to thermocycles on a daily basis. Protection generated during the exposure or recovery from one thermocycle, such as the synthesis of heat-shock proteins, may confer significant protection during subsequent exposure to low or high temperature. This idea is supported by the finding that the cold tolerance of *D. melanogaster* increases with the number of thermocycles to which they are exposed (Kelty and Lee, 2001). Therefore, we believe the role of heat-shock proteins during RCH requires further examination under more ecologically relevant conditions of multiple diurnal thermocycles.

In vitro rapid cold hardening

Recent evidence suggests that organismal survival is matched closely by increases in the cold tolerance of tissues during RCH. Yi and Lee (2004) demonstrated that both *in vivo* and *in vitro* RCH of tissues from *S. crassipalpis* protects cells against cold-shock injury. The survival of isolated tissues (fat body, gut, salivary gland, Malpighian tubules) cold-hardened at 0°C for 2 h significantly increased cell survival (by ~25%) relative to tissues directly exposed to −8°C. We have since confirmed this result in the freeze-tolerant Antarctic midge, *B. antarctica*: RCH hardening of isolated tissues at −5°C, whether frozen or supercooled, for 1 h enhanced cell survival (N.M. Teets, unpublished results). These results are significant as they demonstrate that neuroendocrine mediation from the intact organism is not required to elicit the RCH response; however, it is possible that the nervous and endocrine systems may enhance the cold-hardening effect (Yoder *et al.*, 2006). Additionally, these results suggest a 'cold-sensing' role for the individual cells, as has been demonstrated in plants (see below), in the induction of the cold-hardening response. The fact that individual cells respond directly to low temperature without mediation from higher levels of organization helps to explain the defining characteristic of the RCH response: the swiftness of its induction.

Changes in membrane fluidity and phospholipid composition

Cellular membranes are believed to be the primary sites of cold-shock damage as a result of lipid phase transitions (Drobnis *et al.*, 1993; Hazel, 1995). As temperatures decrease, there is a concomitant decrease in membrane fluidity culminating in a transition from the liquid-crystalline to gel state at which time the membrane loses the ability to function as a selective barrier (Cossins, 1983). However, cells may counter this effect by adjusting the composition of membranes, including the phospholipid head groups and fatty acid chains, and the cholesterol content, to maintain membrane fluidity and the liquid-crystalline state at lower temperatures (Hazel, 1995). This response, termed homeoviscous adaptation (Sinensky, 1974), is well documented in microorganisms, plants and ectothermic animals during acclimation to low temperatures (Los and Murata, 2004).

Recent investigations into the RCH response revealed an increase in the molar percentage of unsaturated phospholipid fatty acids, predominantly linoleic and oleic acid, within the cell membrane at the expense of saturated fatty acids (Overgaard *et al.*, 2005; Michaud and Denlinger, 2006). Additionally, Overgaard *et al.* (2006) documented a similar increase in the degree of membrane unsaturation during an ecologically relevant cooling regime that induces an RCH response in *D. melanogaster*. These changes in membrane composition

would be expected to maintain membrane fluidity at low temperatures and prevent/reduce lipid phase transitions and resulting cellular damage. The significance of these results is further supported by the observation that membrane fluidity increases during RCH (2 h at 0°C) in fat body cells from *Sarcophaga bullata* (Lee *et al.*, 2006a). Taken together, these results suggest that membrane modifications play a vital role in the RCH response protecting against cold-shock injury in insects.

Rapid cold hardening blocks cold-induced apoptosis

Evidence from mammalian cells suggests chilling injury is linked to cold-induced apoptosis (Murakami *et al.*, 1997). Cold shock may induce apoptosis by causing the release of cytochrome-c into the cytoplasm, likely as result of mitochondrial membrane damage, and activation of caspase-3 which initiates the proteolytic cascade ultimately leading to cell death (Cryns and Yuan, 1998). Yi *et al.* (2007) found that a cold shock treatment at −7°C for 2 h induced apoptosis in adult *D. melanogaster*. However, RCH treatment (2 h at 5°C) prior to the low-temperature exposure significantly reduced (by 38%) apoptotic cell death relative to the cold-shocked group. Expression of the anti-apoptosis protein, Bcl-2, perhaps along with heat-shock proteins, may be involved in blocking apoptosis following the RCH treatment (Yi *et al.*, 2007). Further research as to the role of apoptosis in cold-shock injury in insects and the pathway by which RCH prevents such programmed cell death is warranted.

Rapid cold hardening induction pathways

While we are now beginning to gain insight into the physiological mechanisms involved in RCH, the molecular and cellular pathways for induction of the response remain largely unexplored. How is the signal of low environmental temperature 'sensed' and conveyed to induce modification of membrane composition and the synthesis of cryoprotectants ultimately resulting in increased cold tolerance? The early stages of cold acclimation in plants suggest the plasma membrane functions as the primary cold sensor. In lucerne, *Medicago sativa*, cold acclimation is associated with a transient influx of Ca^{2+} and activation of Ca-dependent (CDPKs) and mitogen-activated protein kinases (MAPKs) (Sangwan *et al.*, 2002). Such influx of Ca^{2+} appears to be mediated by low-temperature induced rigidification of the plasma membrane and cytoskeletal rearrangement resulting in the opening of Ca^{2+} channels (Orvar *et al.*, 2000). Similarly to cold acclimation in plants, we recently determined that Ca^{2+} is required to elicit the RCH response in isolated tissues of *B. antarctica* (N.M. Teets, unpublished results). Further, p38 MAPK is activated in *S. crassipalpis* within 10 min of exposure to 0°C (Fujiwara and Denlinger, 2007), a temperature well-known to induce RCH in this species. These results suggest that a similar signalling pathway to that observed in plants is operating in the induction of the RCH response in insects.

Conclusion

For some time now, the RCH response has been known to dramatically increase an insect's protection against cold-shock injury. However, only recently have the broader ecological implications of the response become appreciated, including the reduction of the CT_{min}, thereby extending the range of temperatures over which an insect may remain active, induction of RCH under natural thermal cycles and field conditions, and the preservation of reproductive behaviours and fecundity. RCH therefore appears to be part of a more general hardening response that allows insects to optimize behavioural and physiological performance within a thermally variable environment. Despite the apparent generality of the response among insects, the underlying physiological mechanisms remain poorly understood. While changes in the phospholipid fatty acid composition and the maintenance of membrane fluidity appear to be a component of RCH, the mechanistic role of other physiological changes associated with the response, such as the accumulation of cryoprotective compounds

and the expression of heat-shock proteins, remains inconclusive. Further clarification as to the underlying mechanisms of the response, as well as investigation of the induction pathways of RCH and its role in blocking cold-induced apoptosis, will likely serve as focal points for future research.

Acknowledgements

We thank Ben Philip and Yuta Kawarasaki for their comments on drafts of this chapter. Support from the National Science Foundation, grants #OPP-0337656 and #IOB-0416720, is gratefully acknowledged.

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