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REVIEW

Rapid cold hardening: ecological relevance, physiological mechanisms and new perspectives

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ABSTRACT

Rapid cold hardening (RCH) is a type of phenotypic plasticity that allows ectotherms to quickly enhance cold tolerance in response to brief chilling (lasting minutes to hours). In this Review, we summarize the current state of knowledge of this important phenotype and provide new directions for research. As one of the fastest adaptive responses to temperature known, RCH allows ectotherms to cope with sudden cold snaps and to optimize their performance during diurnal cooling cycles. RCH and similar phenotypes have been observed across a diversity of ectotherms, including crustaceans, terrestrial arthropods, amphibians, reptiles, and fish. In addition to its well-defined role in enhancing survival to extreme cold, RCH also protects against nonlethal cold injury by preserving essential functions following cold stress, such as locomotion, reproduction, and energy balance. The capacity for RCH varies across species and across genotypes of the same species, indicating that RCH can be shaped by selection and is likely favored in thermally variable environments. Mechanistically, RCH is distinct from other rapid stress responses in that it typically does not involve synthesis of new gene products; rather, the existing cellular machinery regulates RCH through post-translational signaling mechanisms. However, the protective mechanisms that enhance cold hardiness are largely unknown. We provide evidence that RCH can be induced by multiple triggers in addition to low temperature, and that rapidly induced tolerance and cross-tolerance to a variety of environmental stressors may be a general feature of stress responses that requires further investigation.

KEY WORDS: Cold tolerance, Ectotherm, Phenotypic plasticity, Rapid cold hardening, Stress

Introduction

Rapid cold-hardening (RCH) (see Glossary), a type of phenotypic plasticity that offers nearly instantaneous protection against acute cold stress in insects, was originally reported in a landmark Science paper more than 30 years ago (Lee et al., 1987). As the name indicates, this response is most vividly distinguished from the process of cold acclimation (see Glossary) by the swiftness of its induction. Cold acclimation, often used in laboratory studies to simulate seasonal cold-hardening, occurs over a course of days to weeks (reviewed by Bowler, 2005), whereas RCH is evident within minutes to hours. For example, in the flesh fly *Sarcophaga crassipalpis* cold shock (see Glossary) at −10°C for 2 h causes >80% mortality; however, when as little as 30 min of exposure to 0°C precedes the same cold shock, mortality decreases to <50% (Lee et al., 1987). RCH is the fastest acclimatory response to low temperature known and is a key adaptation for coping with thermal variability. Daily temperature variation has increased over the last 40 years across many regions of the planet (Dillon et al., 2016) and, therefore, the study of plastic responses, including RCH, will contribute to efforts to predict the effects of climate change on ectotherms (see review by Sgrò et al., 2016).

Debates exist as to whether hardening and acclimation reflect a continuum of the same physiological responses (e.g. Loeschcke and Sørensen, 2005). In the case of RCH and cold acclimation, there are considerable mechanistic differences (see review by Teets and Denlinger, 2013b and discussion below). Moreover, while cold acclimation can be promoted at temperatures conducive to development (e.g. Colinet and Hoffmann, 2012), RCH is generally elicited by temperatures below the developmental threshold, but gradual cold acclimation can also occur below the developmental threshold (see MacMillan et al., 2016). Thus, for the purposes of this Review, we define RCH as beneficial acclimation that occurs within a time course of less than a day, in response to chilling below the developmental threshold. In the following sections, we summarize the ecological relevance, evolutionary genetics and molecular mechanisms of RCH. We also present evidence that RCH may be part of a generalized ability to rapidly adjust stress tolerance in changing environments.

Ecological relevance of RCH

The RCH response is widespread among arthropods and other ectotherms

The list of species that exhibit RCH is extensive. Among insect orders, this response has been observed in Coleoptera, Diptera, Hemiptera, Lepidoptera, Orthoptera and Thysanoptera (see review by Lee and Denlinger, 2010). A notable recent addition to this list is the bumblebee, *Bombus terrestris audax* (Owen et al., 2013), which was the first report of RCH in Hymenoptera. Additionally, RCH is also present in non-insect arthropod taxa, including crustaceans (Ronges et al., 2012), Acari (Broufas and Koveos, 2001; Ghazy and Amano, 2014) and Collembolla (Bahrndorff et al., 2009). Within a species, RCH can occur across developmental stages. For example, in *Drosophila melanogaster*, this response is evident in larval, pupal and adult stages (Czajka and Lee, 1990), whereas in the predatory mite *Neoseiulus californicus* exposure to 5°C for 2 h elicits RCH in all life stages, including eggs (Ghazy and Amano, 2014). RCH can also be induced in individuals that are developmentally programmed for diapause; in *S. crassipalpis*, hardening at 0°C improves cold shock survival in diapause-destined individuals prior to or shortly after pupariation (see Glossary) (Chen et al., 1987). Even among tropical species, some are capable of RCH (see Nyamukondiwa et al., 2011), although it is not universal (e.g. Chen et al., 1990; Terblanche et al., 2008). Therefore, RCH is a widely used adaptation of insects and other related arthropods to cope with thermally variable environments.

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Glossary

Chill coma
A reversible state of paralysis at low temperature caused by neuromuscular impairment stemming from cold-induced membrane depolarization.

Chill coma recovery
The process of regaining locomotor capacity after a nonlethal cold event below the critical thermal minimum.

Chill-susceptible
Describes organisms that are freeze intolerant and succumb to cold at temperature well above the supercooling point.

Cold acclimation
Improved function at low temperature as a result of prolonged (days to weeks) exposure to lower ambient temperature.

Cold shock
A brief exposure to non-freezing low temperature (typically lasting only a few hours at temperature below 0°C) that causes direct chilling injury.

CTmin or critical thermal minimum
The lowest temperature at which an ectotherm can maintain physiological performance, often locomotor function, in the context of acute cold stress.

Cross-talk
When two distinct stressors have overlapping signaling pathways, such that activation of one stress signaling pathways concurrently activates all or part of a distinct stress signaling pathway.

Cross-tolerance
When two distinct stressors share similar protective mechanisms and thus afford protection to one another.

Cryoprotectants
Low molecular weight solutes that accumulate in high quantities in cold tolerant ectotherms to protect against chilling or freezing injury.

Direct chilling injury
Damage to cellular macromolecules (e.g. lipids, proteins) caused by an acute exposure to low temperature in the absence of freezing

Freeze concentration
The process by which extracellular solutes become concentrated during ecologically relevant freezing. When freezing is restricted to extracellular spaces, only water joins the ice lattice, which reduces the amount of liquid water outside the cells and concentrates solutes.

Freeze-intolerant
Describes ectotherms in which internal freezing is lethal.

Freeze-tolerant
Describes a cold tolerance strategy in which an ectotherm can survive internal ice formation.

Homeoviscous adaptation
The process by which adjustments in cell membrane composition promote maintenance of appropriate membrane fluidity at the environmental temperature.

Pupariation
In higher Diptera (flies), the process of forming the puparium, which is a hardened shell derived from the molted larval cuticle that protects the pupa contained within.

Rapid cold-hardening
A process by which ectotherms rapidly enhance their cold tolerance in response to brief (minutes to hours) chilling or another acclimation cue.

Supercooled
Describes a liquid that is cooled below its freezing point without solidification.

Supercooling point
The temperature at which ice crystallization occurs and an organism spontaneously freezes.

RCH-like phenotypic plasticity has also been reported in other ectothermic animals, including some vertebrate species. For example, in the cane toad *Rhinella marina* acclimation to 12°C for 12 h reduces the critical thermal minimum (CT_{min}) (see Glossary) by ∼2°C, compared with those maintained at 24°C (McCann et al., 2014). Also, in tadpoles of the neotropical túngara frog *Engystomops pustulosus* a prior induction of chill coma (see Glossary) by cooling at 1.0°C min^{-1} slightly depresses CT_{min} during a subsequent trial (Vo and Gridi-Papp, 2017). Similarly, some species of fish (Hazel and Landrey, 1988), salamanders (Layne and Claussen, 1987) and turtles (Muir et al., 2010) exhibit RHC-like responses. Thus, although RCH is less well studied outside of arthropods, it is tempting to speculate that rapid phenotypic plasticity at low temperature is a general feature of ectotherms.

The cold tolerance strategy of an ectotherm is traditionally categorized by the ability to tolerate internal ice formation (Lee, 2010). A majority of species are chill-susceptible and freeze-intolerant (see Glossary), and mortality from cold occurs in the absence of internal ice formation well above the supercooling point (see Glossary) as a result of direct chilling injury (see Glossary). Initially, reports of RCH were restricted to chill-susceptible/freeze-intolerant species, and therefore, this response was considered a physiological mechanism to protect cells against direct chilling injury. The first report of RCH in a freeze-tolerant species (see Glossary) was in larvae of the Antarctic midge *Belgica antarctica* (Lee et al., 2006b). Subsequently, the list has been expanded to include freeze-tolerant larvae of another midge species, *Eretmoptera murphyi* (Everatt et al., 2012), and the goldenrod gall fly *Eurosta solidaginis* (Gantz and Lee, 2015; Levis et al., 2012; Teets et al., 2013). Thus, RCH protects not only against direct chilling injury, but also against freezing injury imposed by the combined effects of low temperature and cellular dehydration resulting from freeze concentration (see Glossary) of extracellular fluids (Mazur, 2004). RCH can be elicited in either the frozen or supercooled (see Glossary) state in *B. antarctica*, but protection is greater in frozen larvae (Kawarasaki et al., 2013). When larvae are frozen during RCH, only extracellular water is frozen, and it appears that intracellular processes that regulate RCH (see below) are still active in frozen *B. antarctica*. In contrast, RCH is only observed when larvae are supercooled in *E. murphyi*, indicating that ice formation hinders RCH in this species (Everatt et al., 2012).

Induction of RCH by direct chilling and ecologically relevant cooling

The various ways by which RCH is induced, and its phenotypic outcomes, are summarized in Fig. 1. In laboratory studies, RCH is conventionally induced by an abrupt transfer to a mildly low temperature. The optimal range of temperatures for RCH induction varies among species. For example, in pharate adults (i.e. flies just prior to molting to the adult stage) of *S. crassipalpis*, RCH is most effectively elicited by temperatures between 0 and 10°C (Chen et al., 1987; Fig. 1A). Remarkably, in larvae of *B. antarctica*, whose habitat remains relatively cold year round (see review by Lee and Denlinger, 2015), optimal induction occurs in the subzero range, even while larvae are frozen, and temperatures as low as −12°C effectively promote hardening (Kawarasaki et al., 2013). Although each species has a distinct window of temperatures that triggers RCH, hardening is often elicited efficiently by temperatures around 10°C above the lower lethal temperature (Nyamukondiwa et al., 2011).

Although there was initially concern that RCH may be a laboratory artifact due to the use of unnatural, stepwise temperature transfers, subsequent work has clearly demonstrated its ecological relevance. RCH is also elicited by slow-cooling regimes that mimic natural fluctuations in habitat temperatures (Fig. 1B). For example, in adult *D. melanogaster*, cooling from 23°C to 0°C at 0.1 or 0.05°C min^{-1} promotes RCH and improves cold shock tolerance (Kelty and Lee, 1999). In natural environments, diurnal fluctuations

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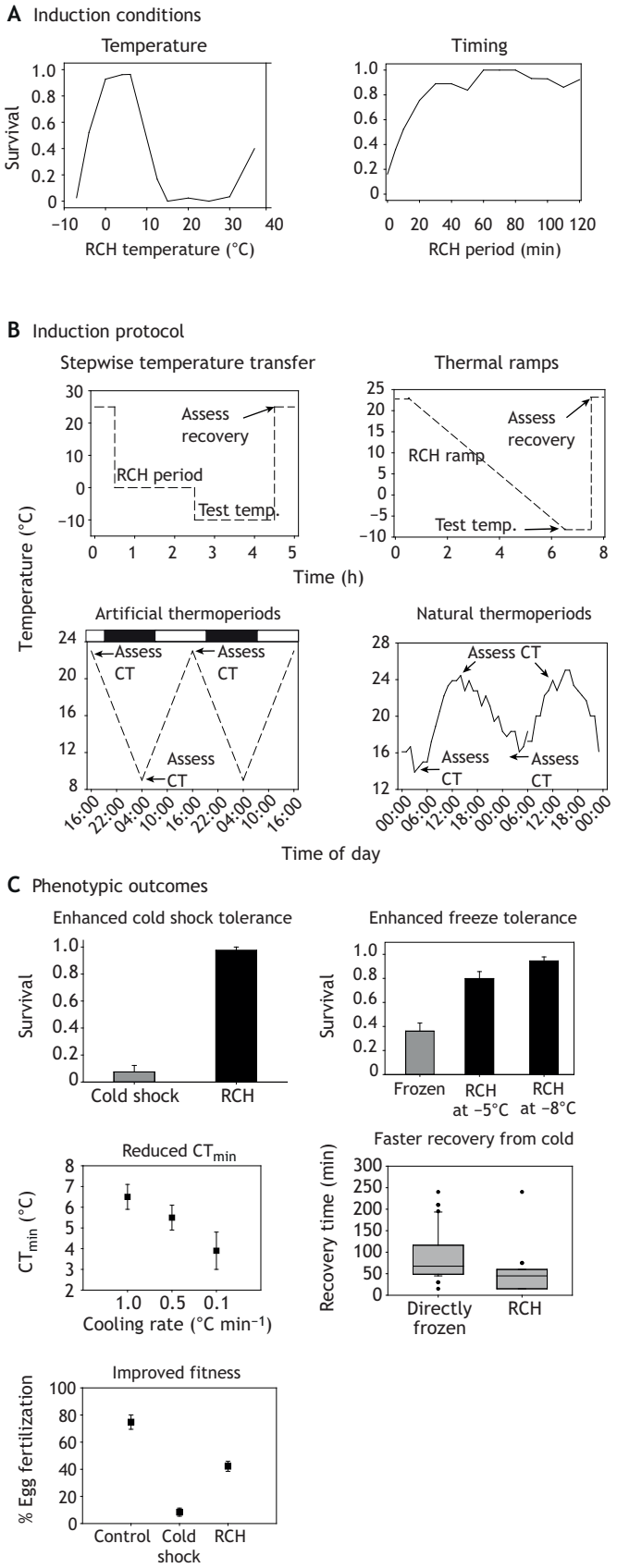

Fig. 1. Summary of the induction conditions and phenotypic outcomes of rapid cold hardening (RCH). (A) The narrow temperature windows and short time periods in which RCH typically occurs. Maximal hardening typically occurs at ∼10°C above LLT_{90} (left). RCH occurs within minutes, with maximal hardening after 1–2 h (right). The graphs show example data that were recreated from Chen et al. (1987). (B) The various temperature protocols that have been used to induce RCH. RCH is most commonly elicited with stepwise temperature transfers, but RCH can also be elicited with thermal ramps, artificial thermoperiods and natural thermoperiods. The graph of natural thermoperiods shows air temperature data for Lexington, KY on 17–18 April 2019. (C) Phenotypic outcomes of RCH. The graphs include example data illustrating the general phenomena; from Teets and Denlinger (2016), Kawarasaki et al. (2013), Kelty and Lee (1999), Teets et al. (2019) and Rinehart et al. (2000), respectively. All figures were adapted with permission from the authors. CT, cold tolerance; CT_{min}, critical thermal minimum temperature; LLT_{90}, lower lethal temperature that induces 90% mortality.

cause gradually decreasing temperatures at night. For example, a natural population of *D. melanogaster* in Michigan, USA (43.60°N, 84.77°W) experiences diurnal cooling from ∼22°C to ∼10°C at a rate of 1.3±0.1°C h^{-1} during the late spring/early summer (Kelty, 2007). When flies are removed from field cages at different times of day, individuals tested at the coldest time of a day (i.e. 06:00 h) are more cold tolerant than those tested at the beginning (i.e. 18:00 h) or middle (i.e. 00:00 h) of the cooling period (Kelty, 2007). A similar field induction of RCH has also been observed in *D. melanogaster* from Denmark (Overgaard and Sørensen, 2008) and the olive fruit fly *Bactrocera oleae* in Greece (Koveos, 2001), and these results indicate that RCH allows insects to track fluctuations in temperature and optimize cold tolerance in real-time.

Notably, protection obtained during RCH is quickly lost upon rewarming. During simulated diurnal thermal regimes, cold hardiness is lost at least partially during the warming phase (Kelty and Lee, 2001), and this observation is consistent with other studies demonstrating the transient nature of RCH (e.g. Chen et al., 1991; Kawarasaki et al., 2013). However, the protective effects of RCH appear to accumulate in response to consecutive thermoperiods (i.e. 24 h of temperature fluctuations that simulate diurnal warming and cooling cycles), as flies become progressively more cold-tolerant when experiencing multiple thermoperiodic cycles (Kelty and Lee, 2001). Additionally, the CT_{min} of these flies is reduced during the first cooling phase, and this enhanced resistance to chilling is maintained through subsequent cycles for up to 7 days, despite the occurrence of warming phases (Kelty and Lee, 2001). Although an increasing number of studies have used slow-cooling regimes to investigate the ecological relevance of RCH, only a handful have incorporated multiple thermocycles (Basson et al., 2012), and the effects of multiple cold exposures cannot always be predicted from those of a single exposure (Marshall and Sinclair, 2010, 2012; Teets et al., 2011). Thus, additional studies involving multiple chilling and rewarming cycles are needed to clarify the cumulative effects of RCH suggested by the early work of Kelty and Lee (2001), since cooling events rarely occur in isolation.

RCH protects against sublethal stress

Although improved survival of extreme cold is a useful measure of RCH induction in laboratory studies, animals may not frequently experience these extreme conditions in natural environments. Thus, assessing the ability of RCH to protect against sublethal cold injury offers additional insights into its ecological benefits (Fig. 1C). Below the CT_{min}, locomotor ability is impaired, but RCH can extend this lower limit of activity. For example, in the migratory locust *Locusta migratoria* chilling at 4°C for 4 h reduces CT_{min} from 7.5±0.1 to 5.1±0.1°C (Srithiphaphirom et al., 2019). Similarly, in *D. melanogaster*, flies cooled at slow rates to induce RCH have a 2–4°C reduction in CT_{min} relative to flies cooled at faster rates (Kelty and Lee, 1999). Similar reductions in CT_{min} are observed in

flies sampled from field cages at different times of the day (Kelty, 2007).

RCH also promotes faster recovery from chill coma in some species (Fig. 1C). For example, in *L. migratoria*, RCH at 0°C reduces the time required to recover from cold shock by approximately 15% (Findsen et al., 2013). However, this effect is not evident in adult *D. melanogaster*, as pre-treatment by chilling at 4.5°C for 3 h or 0°C for 2 h does not affect recovery time from a sublethal cold exposure (Rako and Hoffmann, 2006), suggesting that improved chill coma recovery (see Glossary) is not a general feature of RCH. In larvae of *B. antarctica*, RCH at −5°C for 2 h prior to freezing promotes faster recovery of movement and resumption of metabolic activity compared with larvae directly frozen at a nonlethal temperature of −9°C for 24 h (Teets et al., 2019). In the monarch butterfly *Danaus plexippus* RCH preserves flight behavior after cold stress; chilling at 4°C for 2 h allows more individuals to recover normal flight ability within 24 h after exposure to −4°C, compared with those exposed directly (∼85% vs ∼37%; Larsen and Lee, 1994). Similarly, in *S. crassipalpis*, RCH improves recovery of the proboscis extension reflex (Kelty et al., 1996), as well as retention of spatial discrimination acquired through associative learning (Kim et al., 2005), each of which is severely impaired by cold shock.

Finally, protection by RCH provides energetic and fitness advantages to insects surviving low-temperature stress (Fig. 1C). For example, *B. antarctica* larvae that are directly frozen experience a significant depletion in glycogen stores, whereas those that undergo RCH before freezing are able to preserve their glycogen stores (Teets et al., 2019). In *S. crassipalpis,* cold shock in the pharate adults reduces longevity and >75% of individuals die within 10 days of eclosion; yet, when RCH precedes cold shock, ∼85% remain alive at the same age (Rinehart et al., 2000). Among males that survive to 10 days after eclosion, cold shock negatively impacts fitness, as indicated by a substantial reduction in the rates of successful fertilization from 74.8±5.3% to 8.4±3.0%, but RCH mitigates this loss of fertility and improves fertilization success to 42.2±3.7% (Rinehart et al., 2000). Similarly, in the house fly, *Musca domestica*, sublethal cold exposure at −7°C decreases egg production, possibly because of reduced female lifespan longevity and reduced daily oviposition (egg-laying) in females, and RCH at 0°C improves fecundity (Coulson and Bale, 1992). Even at mildly low temperatures, RCH preserves reproductive success. In adult *D. melanogaster*, courtship and mating behaviors are lost after immediate transfer from 23°C to 16°C, but these functions are restored within 2 h at 16°C (Shreve et al., 2004). Thus, in addition to its well-established role in protecting against mortality from cold, RCH preserves essential ecological functions following sublethal cold stress.

Potential costs associated with RCH

Although RCH has clear benefits in improving performance at low temperatures, several studies have reported that the induction of RCH may impose ecological costs. For example, in *D. melanogaster*, RCH elicited by diurnal cooling reduces heat tolerance, suggesting trade-offs between cold and heat tolerance (Overgaard and Sørensen, 2008). In *D. melanogaster*, adults cold-hardened by slow cooling experience a slight but significant increase in mortality, as well as reduced fecundity during the 8 h period after the treatment, compared with those maintained at the rearing temperature (Overgaard et al., 2007). Chilling at 4°C for 2 h also decreases mating effectiveness in males, indicated by the increased duration of courtship and decreased rates of copulation (Everman et al., 2018). Finally, in the Mediterranean fruit fly *Ceratitis capitata*, repeated daily inductions of RCH by slow cooling increase mortality after 5 days (Basson et al., 2012). However, other studies did not find evidence of trade-offs between RCH and development, longevity or fecundity (Kelty and Lee, 1999; Powell and Bale, 2004, 2005). Thus, future efforts to clarify the ecological costs of RCH are needed to provide insights into its evolution; such research may explain the observed inter- and intraspecific variation in RCH capacity (discussed below; see Gerken et al., 2015; Nyamukondiwa et al., 2011).

Evolutionary genetics of RCH

RCH is reasonably well-studied at the molecular and physiological level (see below), but the evolutionary forces that have shaped RCH across ectotherms have received little attention. Although most insects and other arthropods appear to be capable of RCH (Lee and Denlinger, 2010), the magnitude of hardening varies, and there are species that lack an RCH response altogether (Burks and Hagstrum, 1999; Sinclair and Chown, 2003; Stotter and Terblanche, 2009; Terblanche et al., 2008), indicating that certain environments favor stronger or weaker RCH phenotypes. As a type of adaptive phenotypic plasticity, current hypotheses indicate that RCH and other plastic responses to temperature are likely a critical, yet underappreciated, component of an organism's ability to respond to rapid environmental change (Chevin et al., 2010; Sgrò et al., 2016; Stillman, 2003). Thus, inter- and intraspecific comparisons of RCH and other types of thermal acclimation are needed to predict how these phenotypes will contribute to ectotherm responses to climate change.

To date, only two studies have thoroughly investigated RCH capacity across species and genetically variable lines. Nyamukondiwa et al. (2011) assessed RCH capacity in 18 species of *Drosophila* collected from a variety of environments across three continents. The lower lethal temperature that induces 90% mortality (LLT_{90}) ranges from −3 to −13°C, and a 2 h pretreatment 10°C above the LLT_{90} significantly improves cold tolerance in 15 of the 18 species. Importantly, after controlling for phylogeny, there is a negative relationship between basal cold tolerance and the magnitude of RCH, indicating that hardening capacity may be constrained by basal cold tolerance. In the same study, the opposite pattern was observed for heat hardening, i.e. species with higher heat tolerance also have higher heat hardening capacity (Nyamukondiwa et al., 2011), suggesting a trade-off between basal tolerance and thermal plasticity at low, but not high temperature (also see Kellett et al., 2005). Generally, species with higher levels of basal cold tolerance occur at higher latitudes, so the apparent trade-off between basal tolerance and RCH capacity may be a consequence of the diurnal temperature variation decreasing when moving from temperate to polar regions (Wang and Dillon, 2014).

Predicting how RCH may evolve in response to environmental change also requires a thorough assessment of intraspecific variation in RCH capacity. Gerken et al. (2015) measured the magnitude of RCH across 184 lines from the *Drosophila* genetic reference panel (DGRP), a collection of isogenic lines derived from a single mid-latitude population in Raleigh, North Carolina, USA. There is considerable variation in RCH capacity across these lines; although most lines show improved ability to survive a lethal cold shock, some have no apparent hardening ability, and yet others show a decrease in cold tolerance after pre-treatment. As with interspecific comparisons, RCH capacity is constrained by basal tolerance, with highly cold-tolerant lines having a reduced capacity for hardening. Also, lines with high RCH capacity tend to have

higher capacity for developmental acclimation, suggesting that the two processes may have some mechanistic overlap. However, RCH further enhances cold tolerance in most acclimated flies, and the genetic architectures of RCH and cold acclimation are non-overlapping, indicating each plastic response may have distinct underlying genetic mechanisms (Gerken et al., 2015).

Follow-up studies subjecting subsets of the same DGRP lines used in Gerken et al. (2015) to ecologically relevant cooling ramps also indicate there is significant genetic variation in the ability to harden. However, across genetically distinct lines, RCH capacity elicited by different thermal regimes (i.e. constant temperature, fast ramp and slow ramp) is correlated, suggesting that all three types of RCH share similar underlying mechanisms (Gerken et al., 2018). Yet, the sublethal costs of hardening on courtship and reproduction are similar across genotypes and unrelated to hardening capacity (Everman et al., 2018), indicating that behavioral responses to hardening are independent of thermal tolerance. Furthermore, the persistence of RCH after rewarming is not genetically variable, and in most genotypes the protection afforded by RCH lasts for 2 h after returning flies to 25°C (Everman et al., 2017).

Although the above studies have yielded important insights into the evolutionary physiology of RCH, it is important to highlight some difficulties in performing inter- and intraspecific comparisons of RCH. Identifying appropriate test temperatures is a challenge because there are unlimited combinations of test temperatures, hardening conditions and exposure times that can all influence the estimation of RCH capacity. The studies by Nyamukondiwa et al. (2011) and Gerken et al. (2015) used a test temperature at or close to the LLT_{90} and a hardening temperature 10°C above the LLT_{90}. Although this standardizes the selection of the test temperature and hardening conditions, it assumes all species or genotypes will have an identical, optimal hardening condition. Furthermore, in the DGRP, the relationship between survival and cold shock temperature is genetically variable (Teets and Hahn, 2018), but how this variation in the shape of cold survival curves confounds estimation of hardening capacity has not been addressed. Despite these difficulties in standardizing assays for estimating RCH capacity, the above studies in *Drosophila* clearly indicate that fine-scale intraspecific variation in RCH and broad-scale variation across species must be considered when predicting responses to environmental change.

Physiological and molecular mechanisms of RCH

In a previous review, we detailed physiological mechanisms that are associated with RCH and cold acclimation (Teets and Denlinger, 2013b). Here, we will discuss the current state of knowledge regarding the mechanisms of RCH, with a particular focus on recent developments. A summary of the genes, cell signaling events and biochemical changes that accompany RCH is provided in Table 1. Note this is not intended to be a mechanistic model of RCH, but rather a comprehensive list of the molecular and biochemical processes that have been associated with RCH in various studies. In the paragraphs below, we attempt to synthesize findings from these disparate studies and identify common themes in the regulation of RCH.

Allelic variants associated with RCH

The study by Gerken et al. (2015) using the DGRP also used a genome-wide association study (GWAS) to identify numerous candidate genes associated with RCH. There are 164 single nucleotide polymorphisms (SNPs) associated with RCH capacity, and the genes containing these SNPs are involved in a variety of biological processes including cell death regulation (i.e. autophagy and apoptosis), cell membrane and cytoskeletal dynamics, and redox balance, to name a few. Of these candidate genes, several were functionally validated with mutant strains. Most notably, null mutations in three genes linked to autophagy (*Atg7*, *Eip74EF* and *px*) cause a reduction in RCH capacity, providing the first molecular evidence that autophagy contributes to RCH (Gerken et al., 2015). Autophagy is a cell preservation pathway in which damaged organelles and macromolecules are engulfed and degraded, thereby preventing cell death and conserving energy (He and Klionsky, 2009). Autophagy has been previously associated with desiccation stress in insects (Teets and Denlinger, 2013a) and it has extensive cross-talk with apoptosis, another cell death pathway implicated in RCH (see below; Yi and Lee, 2011; Yi et al., 2007).

Transcriptional regulation of RCH

RCH is one of the fastest known acclimatory responses to thermal stress, rivaling the well-characterized heat shock response (Morimoto, 1998). The heat shock response is largely mediated by upregulation of molecular chaperones to maintain protein homeostasis. Given that cold can cause similar cellular stress, it was initially presumed that RCH would involve an analogous transcriptional program. A microarray analysis in *D. melanogaster* (Qin et al., 2005) reported 37 differentially expressed genes during RCH; however, the authors gave flies a 30 min recovery period after hardening (which is not required to generate hardening), making it impossible to determine whether the observed changes are related to hardening or simply reflect biological responses to cooling and rewarming. In contrast, gene expression measurements taken immediately after the hardening period indicate that RCH generally does not require the synthesis of new gene products. In *D. melanogaster*, candidate genes involved in recovery from cold stress (heat shock proteins and *Frost*) are not differentially expressed during a chilling period that elicits RCH (Sinclair et al., 2007). Subsequent work assessing 219 genes found that none are differentially regulated during RCH in *D. virilis*, whereas only one (*P5cr*) is upregulated (with two downregulated; *Eip71CD* and *cwo*) in the cold-adapted *D. montana* (Vesala et al., 2012). Thus, in *Drosophila* at least, there is no strong evidence that RCH causes transcriptional activation.

More convincing evidence of a lack of transcriptional regulation in RCH was provided in a transcriptomic assessment in the flesh fly *Sarcophaga bullata*, in which the abundance of >15,000 transcripts was quantified. In this species, 2 h of RCH at 0°C causes a dramatic increase in cold tolerance, but the same conditions fail to elicit any changes in gene expression (Teets et al., 2012). Conversely, nearly 10% of the transcriptome (~1500 transcripts) is differentially expressed during recovery from cold stress. Thus, it appears that the short time course (minutes to hours) and low temperatures (typically around 0°C) that trigger RCH do not permit transcriptional activity. Although temperature dependence of RNA polymerase has not been assessed in insects, polymerases from mesophilic bacteria (e.g. *E. coli*) are inactive at 0°C (Uma et al., 1999), so perhaps it is not surprising that RCH fails to produce new transcripts.

Based on the evidence above, previous models suggested that transcriptional regulation is not a component of RCH (Teets and Denlinger, 2013b). However, in recent years, select studies have observed transcriptional changes accompanying RCH. In a few cases, upregulation of heat shock proteins is observed in response to conditions that elicit RCH (Ahn et al., 2018; Lu et al., 2016; Yang et al., 2018). Upregulation of a transcript encoding calcium/

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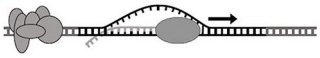
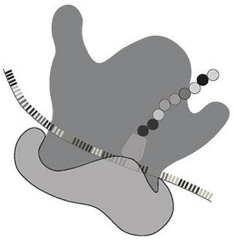
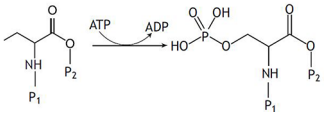
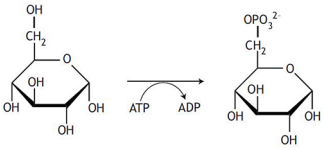
Table 1. Summary of molecular mechanisms associated with rapid cold hardening (RCH) across species

Biological process	Specific mechanism	Gene and protein classes, cell signaling events and biochemical changes	Reference
Allelic variation 	Gene classes associated with variation in RCH	Iron binding, apoptosis*, cell adhesion, calcium binding, cytoskeleton, cuticle, oxidation–reduction, cell membrane*, autophagy*	Gerken et al., 2015
Transcription 	Gene classes upregulated‡	Heat shock proteins, cryoprotectant synthesis, fatty acid metabolism, phosphagen synthesis, cytoskeletal organization, mitochondrial organization, calcium signaling	Ahn et al., 2018; Kim et al., 2017; Lu et al., 2016; Park and Kim, 2014; Vesala et al., 2012; Yang et al., 2018
	Gene classes downregulated‡	Oxidative stress, transcriptional regulation	Vesala et al., 2012
Translation 	Proteins upregulated§	Heat shock proteins, cytoskeletal proteins, fatty acid metabolism, phosphagen synthesis, mitochondrial organization, ribosomal protein, proteolysis, electron transport chain, calcium signaling, nucleotide metabolism	Yang et al., 2018; Li and Denlinger, 2008
	Proteins downregulated§	Heat shock proteins/chaperones, redox balance, proteasome, cytoskeleton, RAS/MAPK signaling, TCA cycle, iron cluster assembly	Li and Denlinger, 2018
Post-translational modifications and cell signaling 	Protein phosphorylation	p38 MAP kinase, Ca^{2+}/calmodulin-dependent protein kinase II, glycogen phosphorylase, multiple proteins involved in: cytoskeleton, cell cycle, morphogenesis, Hippo signaling, phagosome, proteasome, protein processing, mRNA surveillance, endocytosis, carbon metabolism	Fujiwara and Denlinger, 2007; Li et al., 2012; Overgaard et al., 2014; Teets and Denlinger, 2016; Teets et al., 2013
	Apoptotic signaling	Reduced apoptosis and caspase activity	Yi and Lee, 2011; Yi et al., 2007
	Ion signaling and homeostasis	Calcium influx, modulation of K^+ homeostasis following cold shock	Teets et al., 2008; Teets et al., 2013; Armstrong et al., 2012; Findsen et al., 2013
Biochemical changes 	Cell membrane¶	Increase in linoleic acid, oleic acid, overall degree of unsaturation and membrane fluidity	Lee et al., 2006a; Michaud and Denlinger, 2006; Overgaard et al., 2005; Overgaard et al., 2006
	Cryoprotectant accumulation¶	Alanine, glycerol, sorbitol, glucose, trehalose	Michaud and Denlinger, 2007; Overgaard et al., 2007; Park and Kim, 2013; Yoder et al., 2009; Kim et al., 2017; Park and Kim, 2014
	Other metabolic changes	Accumulation of glucose-6-P, fructose-6-P, glutamine, pyruvate and urea; decrease in beta-alanine, ornithine, mannose	Michaud and Denlinger, 2007

*Validated with mutants.
‡Observed in some species but not in well-studied Diptera (see Sinclair et al., 2007; Teets et al., 2012).
§May be an artifact of post-translational modification (see Overgaard et al., 2014; Teets and Denlinger, 2016).
¶But also see MacMillan et al., 2009.
This table is not meant to be a mechanistic model but rather a comprehensive summary of the processes that have been associated with RCH across disparate studies. The illustrations for the DNA double helix and translation were adapted from graphics provided by the Database Center for Life Science under a Creative Commons License (Attribution 4.0 International).

calmodulin protein kinase II, a signaling protein involved in RCH (see below), is detected in the oriental fruit fly *Bactrocera dorsalis* (Ahn et al., 2018). Finally, transcripts encoding metabolic enzymes are also involved in RCH in select species, including transcripts involved in glycerol and trehalose synthesis (Kim et al., 2017; Park and Kim, 2014). Thus, some species appear to have a transcriptional component to RCH, but in well-studied Diptera (i.e. *Drosophila* and flesh flies), a strong RCH response is elicited despite the absence of transcriptional regulation.

Protein synthesis during RCH

Evidence for changes in protein synthesis during RCH largely comes from two proteomic studies. In the brains of *S. crassipalpis*, 38 proteins (out of ∼370 assessed) are differentially abundant between control and RCH flies (Li and Denlinger, 2008). Out of these, 14 were identified by mass spectrometry, and the three proteins upregulated during RCH include ATP synthase, heat shock protein 26 (hsp26) and tropomyosin-1. Downregulated proteins include three proteins involved in proteostasis (including hsp90), three metabolic enzymes and two proteins involved in cytoskeletal dynamics. A similar experiment conducted in whole-body samples of the rice water weevil *Lissorhoptus oryzophilus* reported 21 proteins upregulated and eight downregulated during RCH (Yang et al., 2018). Among the upregulated proteins are two small heat shock proteins, several metabolic enzymes and several proteins involved in cytoskeletal dynamics. Although these two studies suggest that *de novo* protein synthesis is a component of RCH, the results should be interpreted with caution. First, in a later phosphoproteomic analysis of RCH (discussed below), many of the same classes of proteins (and in some cases identical proteins) were also differentially phosphorylated (Teets and Denlinger, 2016). Phosphorylation and other post-translational modifications cause a shift in the isoelectric point, and the shifting of protein spots on a 2D gel can be interpreted as a change in protein abundance (see Overgaard et al., 2014). Second, in *D. melanogaster*, flies are still capable of RCH when protein synthesis is blocked with cycloheximide, calling into question the functional significance of *de novo* protein synthesis (Misener et al., 2001).

Post-translational modifications and cell signaling

The relatively small number of transcripts and proteins synthesized during RCH suggests that RCH is largely regulated by cell signaling. Chilling that induces RCH is accompanied by increased intracellular calcium, and calcium levels track temperature quite closely, suggesting that cells may use calcium to sense temperature and adjust their physiology accordingly (Teets et al., 2008, 2013). Calcium entry into cells is also accompanied by phosphorylation and increased activity of calcium/calmodulin-dependent protein kinase II, although the downstream targets of this signaling enzyme during RCH are unknown. Calcium chelation, blocking calcium channels and antagonizing the calcium-binding protein calmodulin all prevent RCH in tissues (Teets et al., 2008, 2013). Although these experiments indicate a beneficial role of calcium influx during hardening, recent work has also demonstrated that calcium overload is responsible for cell death at low temperature, indicating a dual role for calcium during cold stress (Bayley et al., 2018). Whether calcium leads to beneficial hardening or triggers cell death may depend on its mode of entry into cells and the magnitude of calcium influx.

RCH is also accompanied by rapid phosphorylation of p38 MAP kinase, a stress-inducible kinase involved in many stress responses (Fujiwara and Denlinger, 2007; Li et al., 2012). Within minutes of chilling at 0°C, p38 phosphorylation is detected, and it occurs most strongly at temperatures that elicit RCH (Fujiwara and Denlinger, 2007). Apoptotic signaling is a potential target of p38 during stress, and indeed RCH suppresses apoptotic cell death following cold shock (Yi and Lee, 2011; Yi et al., 2007). This reduction in programmed cell death is accompanied by a significant reduction in the activity of caspases, a group of endoproteases involved in the execution of apoptosis; however, whether this suppression of caspases is directly caused by RCH or is a result of preventing cell damage after cold shock is unclear. Overgaard et al. (2014) also observed apparent phosphorylation of glycogen phosphorylase during RCH, which is accompanied by a slight increase in glucose levels. However, somewhat paradoxically, this phosphorylation is not accompanied by a detectable increase in enzyme activity (Overgaard et al., 2014). Activity was only measured *in vitro*, which may explain the discrepancy between the proteomics and enzyme activity data.

To further identify phosphorylation changes that accompany RCH, Teets and Denlinger (2016) conducted a quantitative phosphoproteomic analysis of fat body and brain tissue from *S. bullata* following RCH. In the fat body and brain, 64 and 82 proteins, respectively, are differentially phosphorylated when tissues are chilled at 0°C for 2 h. Thus, relative to previous studies of mRNA and protein expression, there is an abundance of post-translational change following RCH. Of these differentially phosphorylated proteins, nine are common to both tissues, including three involved in stress responses [l(2)37Cc, Grasp65 and 14-3-3ζ], and several others involved in cytoskeletal dynamics. Among all differentially phosphorylated proteins, the gene ontology term 'response to stress' is enriched, and this term includes three heat shock proteins that are differentially phosphorylated during RCH. Thus, even though RCH is not accompanied by a classic heat shock response, we speculate that differential phosphorylation changes the chaperone activity and/or cellular localization of heat shock proteins during RCH. In addition to stress proteins, proteins involved in cytoskeletal dynamics, vesicle-mediated transport and cell morphogenesis are differentially phosphorylated, indicating that cell structural modifications are an important component of RCH. Finally, KEGG enrichment analysis identified several new pathways involved in RCH, including Hippo signaling, protein processing pathways (proteasome and endoplasmic reticulum) and carbon metabolism (Teets and Denlinger, 2016). Although this study confirmed the likely importance of post-translational modification in RCH and identified new candidates, the functional significance of these protein phosphorylation changes is unclear, and further validation is required to confirm their role in RCH.

In addition to calcium signaling, RCH also modulates potassium homeostasis. Current models of chilling injury indicate that cold-induced membrane depolarization leads to hyperkalemia, and this disruption of ion balance is a major contributor to cold injury (Overgaard and MacMillan, 2017). In the brain of *D. melanogaster*, cold stress causes a dramatic increase in extracellular potassium concentration, and interestingly, flies pretreated with RCH experience an even bigger disruption in potassium homeostasis (Armstrong et al., 2012). However, RCH also allows for faster clearance of potassium during recovery (Armstrong et al., 2012), which may explain the protective effect of RCH despite the larger disruption in ion balance. Nearly identical results were observed in the locust *Locusta migratoria*, in which RCH increases the degree of cold-induced hyperkalemia but allows for faster recovery of homeostasis (Findsen et al., 2013). While the mechanisms allowing faster clearance are unclear, in our previous phosphoproteomics analysis of RCH, we observed differential phosphorylation of many proteins

involved in transport, cytoskeletal dynamics and cellular energetics, all of which may affect ion clearance (Teets and Denlinger, 2016).

Biochemical changes during RCH

Dating back to pioneering work by Salt in the 1950s and 1960s, cryoprotectant synthesis is perhaps the best-studied mechanism by which ectotherms seasonally enhance cold tolerance (Salt, 1961). Several studies have tested the hypothesis that RCH induces cryoprotectant synthesis. In a metabolomic screen of RCH in *S. crassipalpis*, the concentrations of five metabolites increased during hardening, including those of canonical cryoprotectants glycerol and sorbitol (Michaud and Denlinger, 2007). However, the authors elected for RCH treatment lasting 8 h, which is substantially longer than the duration required for maximal hardening in this species (1–2 h; Chen et al., 1987), making it unclear whether the observed changes are necessary for RCH. Indeed, subsequent work in closely related *S. bullata* found no evidence of cryoprotectant synthesis following a 2 h hardening treatment that dramatically improves cold tolerance (Teets et al., 2012). In *D. melanogaster*, RCH elicits a slight but significant increase in glucose and trehalose (Overgaard et al., 2007), although other work has reported an absence of glucose synthesis during RCH (MacMillan et al., 2009). In several moth species, increased levels of the cryoprotectants glycerol or trehalose are observed during RCH (Kim et al., 2017; Park and Kim, 2013, 2014), providing further evidence that RCH involves cryoprotectant synthesis in certain species. However, the levels of cryoprotectants observed during RCH are substantially lower than those typically observed during seasonal cold acclimation, calling into question how these biochemical changes contribute to cold hardening.

Another biochemical change that accompanies RCH is the modification of cell membranes. The hypothesis of homeoviscous adaptation (see Glossary) indicates that organisms adjust the composition of their cell membranes to maintain fluidity at different temperatures (Sinensky, 1974), and this process has been identified as an important component of cold hardiness (Koštál, 2010). In both *D. melanogaster* and *S. crassipalpis*, RCH causes an increase in the abundance of certain unsaturated fatty acids and an overall increase in the degree of unsaturation (Michaud and Denlinger, 2006; Overgaard et al., 2005), although the rate of cooling influences the exact nature of cell membrane modifications (Overgaard et al., 2006). These changes in membrane composition result in measurable increases in membrane fluidity when intact membranes are measured with 31P solid-state NMR (Lee et al., 2006a). However, as with cryoprotectant accumulation, there are cases where RCH is observed in the absence of any detectable changes in cell membrane composition (MacMillan et al., 2009).

Towards a mechanistic understanding of RCH

As detailed above, many genes and pathways have been linked to RCH in studies spanning levels of biological organization, but this information was obtained from numerous species using a variety of methodologies. Thus, the key mechanisms that protect against cold injury during RCH are still largely unknown. However, some important patterns, including the importance of cell signaling and post-translational modifications, are emerging from these disparate studies, and we recommend the following future directions. Firstly, there is discrepancy over the role of transcription in RCH, and all the studies that have observed transcriptional changes were in Lepidoptera, which could indicate taxon-specific mechanisms for RCH. Carefully designed, comparative transcriptomic experiments across species would help clarify the role of gene expression in RCH. Secondly, while cell signaling is an important regulator of RCH, most of the cell signaling work to date has been conducted in non-model species, so moving this research to model species (i.e. *D. melanogaster*) may help advance these ideas. Finally, for any mechanistic study of RCH, we recommend using the minimal duration of cold exposure that elicits a maximal RCH response, and a careful study design to differentiate the physiological processes activated during hardening versus those activated during recovery. The increased availability of reverse genetic techniques (e.g. RNAi and genome editing) will also help to clarify the mechanisms that directly contribute to RCH.

We recommend the following steps to select RCH conditions for mechanistic studies. (1) Empirically determine the LLT_{90} for your species of interest. The time of cold exposure is somewhat arbitrary, but brief exposures lasting for 1–2 h are reasonable, since daily minimum temperatures are typically experienced within this timeframe. (2) Limit the duration of acclimation to the minimum time required to induce maximal hardening. For most species, maximal hardening occurs within 1–2 h. Thus, we recommend starting with a 1 h hardening period and increasing the duration as needed to achieve maximal hardening. (3) Empirically determine the optimal temperature for RCH induction. Use of a temperature 10°C above the LLT_{90} generally works well to elicit hardening in most species. If no hardening is observed at this temperature, attempt additional temperatures within ∼5°C above and below this temperature. (4) Use the empirically determined RCH conditions from Steps 2 and 3 for physiological and molecular experiments. Using the minimal duration of hardening that elicits a strong phenotypic response will reduce false positives and increase the chances of identifying mechanisms that are necessary and sufficient for RCH.

Multiple triggers elicit RCH

Earlier in the review, we defined RCH as acclimation that occurs in less than a day in response to chilling below the developmental threshold; however, it is becoming increasingly clear that RCH is also elicited by other cues (Table 2). Exposure to high temperature,

Table 2. Summary of triggers that elicit rapid acclimation within 24 h and their effects on stress tolerance

Pretreatment condition	Effect	Reference
Chilling	↑ Cold tolerance	Lee et al., 1987 and numerous others
	↑ Freezing tolerance	Kawarasaki et al., 2013
	↑ Dehydration resistance	Yoder et al., 2006
	↑ Anoxia tolerance	Gantz et al., 2020b preprint
	↑ Immune system activity	Salehipour-shirazi et al., 2017
Freezing	↑ Freezing tolerance	Lee et al., 2006b; Kawarasaki et al., 2013
High temperature	↑ Cold tolerance	Chen et al., 1991
Dehydration	↑ Cold tolerance	Yi et al., 2017; Kawarasaki et al., 2019
	↑ Freezing tolerance	Levis et al., 2012
	↑ Dehydration resistance	Hoffmann, 1990; Bazinet et al., 2010
Hyperosmotic stress	↑ Freezing tolerance	Gantz et al., 2020b preprint
Hypo-osmotic stress	↑ Freezing tolerance	Gantz et al., 2020b preprint
Anoxia	↑ Cold tolerance	Coulson and Bale, 1991
Fasting	↑ Freezing tolerance	Gantz et al., 2020b preprint
UV irradiation	↑ Freezing tolerance	Gantz et al., 2020b preprint

anoxia and dehydration, in particular, can cause measurable increases in cold tolerance within 1–2 h (Chen et al., 1991; Coulson and Bale, 1991; Gantz and Lee, 2015; Kawarasaki et al., 2019; Levis et al., 2012). Similarly, other stresses, such as fasting and UV irradiation can trigger acquisition of increased cold tolerance within 24 h (Andersen et al., 2013; Gantz et al., 2020b preprint; Le Bourg, 2013). Furthermore, similar to other cues enhancing cold tolerance, mild chilling can enhance tolerance to other stressors, such as anoxia, dehydration and upregulated immune activity (Gantz et al., 2020a; Salehipour-Shirazi et al., 2017; Yoder et al., 2006). This ability of multiple triggers to elicit RCH is analogous to the well-studied phenomenon of hormesis, where mild exposure to stress (e.g. thermal stress, anoxia, insecticides, UV irradiation) has long-lasting impacts on longevity, fitness and stress tolerance (see Calabrese, 2013; Cutler and Guedes, 2017; López-Martínez et al., 2014; López-Martínez and Hahn, 2012; Patil et al., 1996; Scannapieco et al., 2007).

From an ecological perspective, stressful conditions often occur concomitantly (Holmstrup et al., 2010), which may explain the ability of multiple cues to enhance cold hardiness. For example, cold fronts typically produce reduced humidity (Miles, 1962; Moeller et al., 1993), exposure to ultraviolet light may accompany high temperatures and hypoxia can coincide with hypo-osmotic stress during flooding (Hoback et al., 1998). Nevertheless, most studies of RCH have investigated a single cue at a time; simultaneous use of multiple stressors would strengthen our understanding of the ecological relevance of RCH and other rapid acclimatory responses. In the limited data available, multiple stressors can induce more robust hardening responses. For example, in adult flesh flies (*S. bullata*), sequential exposure to chilling and dehydration promotes faster recovery from chill coma than exposure to chilling or dehydration only (Yi et al., 2017). Similarly, when larvae are sequentially exposed to dehydration and chilling, rates of pupariation and cell survival after cold shock are dramatically improved (Yi et al., 2017). In contrast, combinations of other pretreatments, such as nutrient restriction with dehydration or anoxia with chilling, impose negative effects on cold tolerance (Mitchell et al., 2017; Nilson et al., 2006). Although the generality of these results remains to be seen, they suggest that certain stressors trigger shared mechanistic pathways, whereas others elicit distinct protective responses that are incompatible.

Although the mechanisms of RCH are the subject of intense investigation (see above), the physiological and molecular mechanisms triggered by other hardening cues have not been assessed. Chilling and other cues often enhance stress tolerance on similar timescales, i.e. protective effects are seen within 1 h and reach a maximum by ∼2 h after induction (Coulson and Bale, 1991; Gantz and Lee, 2015; Kawarasaki et al., 2019; Lee et al., 1987). Thus, it is tempting to speculate that cold stress and these disparate cues share core signaling pathways (cross-talk; see Glossary) and/or protective mechanisms (cross-tolerance; see Glossary). Indeed, cold and desiccation stress, for example, share many features at the cellular level, and cross-tolerance is often observed between these stressors (Sinclair et al., 2013). However, interactions among different environmental cues that induce hardening responses may be far more complex. In a recent study, we systematically examined rapid cross-tolerance in larvae of *B. antarctica* by exposing larvae to six different acclimation treatments and four different stress conditions in a full factorial design. Here, only certain combinations of stressors elicited cross-tolerance, and there was no clear pattern regarding which cues enhanced tolerance to which stressors. For example, acclimation in an acidic environment (pH 3) for 2 h increased freezing (−14°C for 24 h) and dehydration (35% relative humidity for 24 h) tolerance, but decreased heat tolerance at 30°C and survival of a hyperosmotic challenge in 3.0 mol l^{-1} NaCl solution (Gantz et al., 2020b preprint). Thus, while there is some cross-talk and/or cross-tolerance between various stressors, there also appear to be stress-specific signaling mechanisms, as indicated by the result that no single pre-treatment elicited increased tolerance to every post-treatment. Therefore, systematic investigations of the mechanistic interactions between these stressors offers an exciting opportunity for future research.

Conclusions

The discovery of RCH (Lee et al., 1987) led to a paradigm shift in how we think about cold hardening. Although seasonal adaptations to gradual changes in temperature (i.e. diapause and cold acclimation) were well appreciated, it is now clear that cold tolerance is a flexible trait that can rapidly change in response to temperature and other environmental signals. From the limited studies that have addressed the evolutionary biology of RCH, there are both inter- and intraspecific variation in RCH capacity that is likely shaped by selection, and it appears that the capacity for RCH is constrained by basal cold hardiness. Although the ecological relevance of RCH is well established, the physiological mechanisms have remained somewhat of a puzzle. The mechanisms of chilling injury are becoming increasingly well characterized (Overgaard and MacMillan, 2017), but the protection elicited by RCH does not seem to be determined by canonical cryoprotective pathways (e.g. cryoprotectants, stress protein expression), at least in most cases. Some important upstream regulators (i.e. calcium signaling, p38 MAPK) have been identified, but the downstream processes that confer cold tolerance is an area ripe for further investigation. Furthermore, RCH can be induced by multiple cues besides chilling, but many of these cues are not well characterized. Although the underpinning mechanisms and ecological relevance are unclear, these observations of rapid cross-tolerance provide a new area of research, and in future work, we aim to identify the signaling mechanisms (cross-talk) and protective mechanisms (cross-tolerance) that promote rapid acclimation across stressors. Although this work is in its infancy, it appears that RCH may be part of a collection of generalized rapid acclimation responses that allow organisms to integrate complex environmental signals and optimize performance in temporally variable environments.

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Competing interests

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