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

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Summary Statement

Rapid cold hardening allows ectotherms to quickly enhance their cold tolerance. Here we summarize the ecological relevance, underlying mechanisms, and future research directions for this important process.

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 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 like locomotion, reproduction, and energy balance following cold stress. 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 posttranslational signaling mechanisms. However, the protective mechanisms that enhance cold hardiness are largely unknown. At the conclusion of the Review, 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.

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 two hours 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 impacts of climate change on ectotherms (see review by Sgro et al., 2016).

Debates exist as to whether hardening and acclimation reflect a continuum of the same physiological responses (e.g. Loeschcke and Sorensen, 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 (NB: gradual cold acclimation can also occur below the developmental threshold; see MacMillan et al., 2016 for an example). 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 two hours 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

94 species, some are capable of RCH (see Nyamukondiwa et al., 2011), although it is not universal
95 (e.g. Chen et al., 1990; Terblanche et al., 2008). Therefore, RCH is a widely used adaptation of
96 insects and other related arthropods to cope with thermally variable environments.
97 RCH-like phenotypic plasticity has also been reported in other ectothermic animals,
98 including some vertebrate species. For example, in the cane toad, *Rhinella marina*, acclimation
99 to 12°C for 12 hours reduces the critical thermal minimum (i.e. CT_{min}) (see Glossary) by ~2°C,
100 compared to those maintained at 24°C (McCann et al., 2014). Also, in tadpoles of the neotropical
101 túngara frog, *Engystomops pustulosus*, a prior induction of chill coma (see Glossary) by cooling
102 at 1.0°C min^{-1} slightly depresses CT_{min} during a subsequent trial (Vo and Gridi-Papp, 2017).
103 Similarly, some species of fish (Hazel and Landrey, 1988), salamanders (Layne and Claussen,
104 1987) and turtles (Muir et al., 2010) exhibit RHC-like responses. Thus, although RCH is
105 understudied outside of arthropods, it is tempting to speculate that rapid phenotypic plasticity at
106 low temperature may be a general feature of ectotherms.
107 The cold tolerance strategy of an ectotherm is traditionally categorized by the ability to
108 tolerate internal ice formation (Lee, 2010). A majority of species are chill-susceptible and freeze-
109 intolerant (see Glossary), and mortality from cold occurs in the absence of internal ice formation
110 well above the supercooling point (see Glossary) due to direct chilling injury (see Glossary).
111 Initially, reports of RCH were restricted to chill-susceptible/freeze-intolerant species, and
112 therefore, this response was considered a physiological mechanism to protect cells against direct
113 chilling injury. The first report of RCH in a freeze-tolerant species (see Glossary) was in larvae
114 of the Antarctic midge, *Belgica antarctica* (Lee et al., 2006b). Subsequently, the list has been
115 expanded to include freeze-tolerant larvae of another midge species, *Eretmoptera murphyi*
116 (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 way RCH is inducted, and its phenotypic outcomes, are summarized in Figure 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 10 and 0°C (Chen et al., 1987; Fig. 1). 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 triggerss 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. 1). 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 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. 6:00) are more cold tolerant than those tested at the beginning (i.e. 18:00) or middle (i.e. 00:00) of the cooling period (Kelty, 2007). A similar field-induction of RCH has also been observed in *D. melanogaster* from Denmark (Overgaard and Sorensen, 2008) and 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 required 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 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; Marshall and Sinclair, 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. 1). 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 four hours 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. 1). For example, in *L. migratoria,* RCH at 0°C reduces the time required to recover from cold shock by

185 approximately 15% (Findsen et al., 2013). However, this effect is not evident in adult *D.*
186 *melanogaster*, as neither pre-treatments of chilling at 4.5°C for three hours nor 0°C for two hours
187 affect recovery time from a sublethal cold exposure (Rako and Hoffmann, 2006), suggesting that
188 improved chill coma recovery (see Glossary) is not a general feature of RCH. In larvae of *B.*
189 *antarctica,* RCH at -5°C for two hours prior to freezing promotes faster recovery of movement
190 and resumption of metabolic activity compared to larvae directly frozen at a nonlethal
191 temperature of -9°C for 24 hours (Teets et al., 2019). In the monarch butterfly, *Danaus*
192 *plexippus*, RCH preserves flight behavior after cold stress; chilling at 4°C for two hours allows
193 more individuals to recover normal flight ability within 24 hours after exposure to -4°C,
194 compared to those exposed directly (~85% versus ~37%; Larsen and Lee, 1994). Similarly, in *S.*
195 *crassipalpis,* RCH improves recovery of the proboscis extension reflex (Kelty et al., 1996), as
196 well as retention of spatial discrimination acquired through associative learning (Kim et al.,
197 2005), each of which is severely impaired by cold shock.
198 Finally, protection by RCH provides energetic and fitness advantages to insects surviving
199 low-temperature stress (Fig. 1). For example, in *B. antarctica,* larvae that are directly frozen
200 experience a significant depletion in glycogen stores, whereas those that undergo RCH before
201 freezing are able to preserve their glycogen stores (Teets et al., 2019). In *S. crassipalpis,* cold
202 shock in the pharate adults reduces longevity and >75% of individuals die within ten days after
203 eclosion; yet, when RCH precedes cold shock, ~85% remain alive at the same age (Rinehart et
204 al., 2000). Among males that survive to ten days after eclosion, cold shock negatively impacts
205 fitness, as indicated by a substantial reduction in the rates of successful fertilization from 74.8 ±
206 5.3% to 8.4 ± 3.0%, but RCH mitigates this loss of fertility and improves fertilization success to
207 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 due to 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 two hours 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 Sorensen, 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 eight hour period after the treatment, compared to those maintained at the rearing temperature (Overgaard et al., 2007). Chilling at 4°C for two hours also decreases mating effectiveness in males, indicated by 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; Powell and Bale, 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

Although RCH is reasonably well-studied at the molecular and physiological level (see below), the evolutionary forces that have shaped RCH across ectotherms have received little attention. Though 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 organisms' ability to respond to rapid environmental change (Chevin et al., 2010; Sgro 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 two hour 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 tradeoff 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 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 nonoverlapping, indicating each plastic response may have distinct underlying genetic mechanisms (Gerkan 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, RCH capacity across different thermal regimes (i.e. constant

277 temperature, fast ramp and slow ramp) is genetically correlated, suggesting that all three types of
278 RCH share similar underlying mechanisms (Gerken et al., 2018). Yet, the sublethal costs of
279 hardening on courtship and reproduction are similar across genotypes and unrelated to hardening
280 capacity (Everman et al., 2018), indicating that behavioral responses to hardening are
281 independent of thermal tolerance. Furthermore, the persistence of RCH after rewarming is not
282 genetically variable, and in most genotypes the protection afforded by RCH lasts for two hours
283 after returning flies to 25°C (Everman et al., 2017).

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

299

Physiological and molecular mechanisms of RCH

In our previous review, we detailed physiological mechanisms that are associated with RCH and cold acclimation (Teets and Denlinger, 2013b). Here, we will summarize 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 are found in Figure 2. Note this figure 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 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, reduced expression of three genes linked to autophagy (*Atg7*, *Eip74EF,* and *px*) causes 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 analagous 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,* 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, two hours 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., 2012a). Conversely, nearly 10% of the transcriptome (~1,500 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/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

366 Evidence for changes in protein synthesis during RCH largely comes from two proteomic
367 studies. In the brains of *S. crassipalpis,* 38 proteins (out of ~370 assessed) are differentially
368 abundant between control and RCH flies (Li and Denlinger, 2008). Out of these, 14 were
369 identified by mass spectrometry, and the three proteins upregulated during RCH include ATP
370 synthase, heat shock protein 26 (hsp26) and tropomyosin-1. Downregulated proteins include
371 three proteins involved in proteostasis (including hsp90), three metabolic enzymes and two
372 proteins involved in cytoskeletal dynamics. A similar experiment conducted in whole body
373 samples of the rice water weevil *Lissorhoptus oryzophilus* reported 21 upregulated and 8
374 downregulated proteins during RCH (Yang et al., 2018). Among the upregulated proteins are two
375 small heat shock proteins, several metabolic enzymes and several proteins involved in
376 cytoskeletal dynamics. Although these two studies suggest that *de novo* protein synthesis is a
377 component of RCH, the results should be interpreted with caution. First, in a later
378 phosphoproteomic analysis of RCH (discussed below), many of the same classes of proteins (and
379 in some cases identical proteins) were also differentially phosphorylated (Teets and Denlinger,
380 2016). Phosphorylation and other posttranslational modifications cause a shift in the isoelectric
381 point, and the shifting of protein spots on a 2D gel can be interpreted as a change in protein
382 abundance (see Overgaard et al., 2014). Second, in *D. melanogaster,* flies are still capable of
383 RCH when protein synthesis is blocked with cycloheximide, calling into question the functional
384 significance of *de novo* protein synthesis (Misener et al., 2001).

385

386 ***Post-translational modifications and cell signaling***

387 The relatively small number of transcripts and proteins synthesized during RCH suggests
388 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; Teets et al., 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; Teets et al., 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 the mode of entry into cells and 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,

412 this phosphorylation is not accompanied by a detectable increase in enzyme activity (Overgaard
413 et al., 2014). Activity was only measured *in vitro,* which may explain the discrepancy between
414 the proteomics and enzyme activity data.

415 To further identify phosphorylation changes that accompany RCH, Teets and Denlinger
416 (2016) conducted a quantitative phosphoproteomic analysis of fat body and brain tissue from *S.*
417 *bullata* following RCH. In the fat body and brain, 64 and 82 proteins, respectively, are
418 differentially phosphorylated when tissues are chilled at 0°C for two hours. Thus, relative to
419 previous studies of mRNA and protein expression, there is an abundance of posttranslational
420 change following RCH. Of these differentially phosphorylated proteins, nine are common to both
421 tissues, including three involved in stress responses (l(2)37Cc, Grasp65, and 14-3-3ζ), and
422 several others involved in cytoskeletal dynamics. Among all differentially phosphorylated
423 proteins, the gene ontology term "response to stress" is enriched, and this term includes three
424 heat shock proteins that are differentially phosphorylated during RCH. Thus, even though RCH
425 is not accompanied by a classic heat shock response, we speculate that differential
426 phosphorylation changes the chaperone activity and/or cellular localization of heat shock
427 proteins during RCH. In addition to stress proteins, proteins involved in cytoskeletal dynamics,
428 vesicle-mediated transport and cell morphogenesis are differentially phosphorylated, indicating
429 that cell structural modifications are an important component of RCH. Finally, KEGG
430 enrichment analysis identified several new pathways involved in RCH, including Hippo
431 signaling, protein processing pathways (proteasome and endoplasmic reticulum) and carbon
432 metabolism (Teets and Denlinger, 2016). Although this study confirmed the likely importance of
433 post-translational modification in RCH and identified new candidates, the functional significance

434 of these protein phosphorylation changes is unclear, and further validation is required to confirm
435 their role in RCH.

436 In addition to calcium signaling, RCH also modulates potassium homeostasis. Current
437 models of chilling injury indicate that cold-induced membrane depolarization leads to
438 hyperkalemia, and this disruption of ion balance is a major contributor to cold injury (Overgaard
439 and MacMillan, 2017). In the brain of *D. melanogaster,* cold stress causes a dramatic increase in
440 extracellular potassium concentration, and interestingly, flies pretreated with RCH experience an
441 even bigger disruption in potassium homeostasis (Armstrong et al., 2012). However, RCH also
442 allows for faster clearance of potassium during recovery (Armstrong et al., 2012), which may
443 explain the protective effect of RCH despite the larger disruption in ion balance. Nearly identical
444 results were observed in the locust *Locusta migratoria,* in which RCH increases the degree of
445 cold-induced hyperkalemia but allows for faster recovery of homeostasis (Findsen et al., 2013).
446 While the mechanisms allowing faster clearance are unclear, in our previous phosphoproteomics
447 analysis of RCH, we observed differential phosphorylation of many proteins involved in
448 transport, cytoskeletal dynamics and cellular energetics, all of which may affect ion clearance
449 (Teets and Denlinger, 2016).

450

451 ***Biochemical changes during RCH***

452 Dating back to pioneering work by Salt in the 1950's and 1960's, cryoprotectant
453 synthesis is perhaps the best-studied mechanism by which ectotherms seasonally enhance cold
454 tolerance (Salt, 1961). Several studies have tested the hypothesis that RCH induces
455 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 eight hours, which is substantially longer than the duration required for maximal hardening in this species (one to two hours; 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 two hour hardening treatment that dramatically improves cold tolerance (Teets et al., 2012b). 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; Park and Kim, 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 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 one to two hours 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 one to two hours. Thus, we recommend starting with a one our 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 the 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 1). Exposure to high temperature, anoxia and dehydration, in particular, can cause measurable increases in cold tolerance within one to two hours (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 hours, (Andersen et al., 2013; Gantz et al., in preparation; Le Bourg, 2013). Furthermore, similar to other cues enhancing cold tolerance, mild

chilling can enhance tolerance to other stressors, such as anoxia, and dehydration and upregulated immune activity (Gantz et al., in review; 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) have long-lasting impacts on longevity, fitness, and stress tolerance (see Calabrese, 2013; Cutler and Guedes, 2017; Lopez-Martinez et al., 2014; Lopez-Martinez 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 hypoosmotic 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 one hour and reach a maximum by about two hours 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 companion paper published concurrently with this Review, 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 two hours increased freezing (-14°C for 24 h) and dehydration (35% relative humidity for 24 hours) tolerance, but decreased heat tolerance at 30°C and survival of a hyperosmotic challenge in 3.0 M NaCl solution (Gantz et al., in preparation). 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 are a ripe area of investigation. Further, 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

591 of a collection of generalized rapid acclimation responses that allow organisms to integrate
592 complex environmental signals and optimize performance in temporally variable environments.

593 **Acknowledgements**

594 The authors appreciate the efforts of Stefan Galander and Julian Dow in soliciting and approving
595 submission of this review. We appreciate the through review and editorial comments provided by
596 Dr. Galander and two anonymous reviewers.

597

598 **Competing Interests**

599 The authors declare no competing interests

600

601 **Funding**

602 This work is supported by Hatch Project 1010996 from the USDA National Institute of Food and
603 Agriculture and National Science Foundation grant OIA-1826689 to N.M.T.

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Figure Legends

Figure 1. Summary of the induction conditions and phenotypic outcomes of RCH. The left panel illustrates the narrow temperature windows and short time periods in which RCH typically occurs. The graphs show example data that was recreated from Chen et al. (1987). The middle panel shows 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 April 17-18, 2019. The right panel shows some phenotypic outcomes of RCH. The graphs include example data illustrating the general phenomena; graph 1 is from Teets et al. (2016), graph 2 is from Kawarasaki et al. (2013), graph 3 is from Kelty and Lee (1999), graph 4 is from Teets et al. (2019), and graph 5 is from Rinehart et al. (2000). All figures were adapted with permission from the authors.

Figure 2. Summary of molecular mechanisms associated with RCH across species. The schematics on the left illustrate the various levels of organization that participate in the induction of RCH, and the boxes to the right summarize the gene and protein classes, cell signaling events and biochemical changes that accompany RCH. This figure isn't meant to be a mechanistic model but rather a comprehensive summary 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). Superscripts indicate references that support each part of the figure. [1]Gerken et al., 2015; [2]Ahn et al., 2018; [3]Kim et al., 2017; [4]Lu et al., 2016; [5]Park et al., 2014; [6]Vesala et al., 2012; [7]Yang et al., 2018; [8]Sinclair et al., 2007; [9]Teets et al., 2012; [10]Li and Denlinger, 2008; [11]Overgaard et al., 2014; [12]Teets and Denlinger, 2016; [13]Armstrong et al., 2012; [14]Findsen et al., 2013; [15]Fujiwara and Denlinger, 2007; [16]Li et al., 2012; [17]Overgaard et al., 2014;

[18]Teets and Denlinger, 2016; [19]Teets et al., 2008; [20]Teets et al., 2013; [21]Yi and Lee, 2011; [22]Yi et al., 2007; [23]Lee et al., 2006; [24]Michaud and Denlinger, 2006; [25]Michaud and Denlinger, 2007; [26]Overgaard et al., 2007; [27]Overgaard et al., 2005; [28]Overgaard et al., 2006; [29]Park and Kim, 2013; [30]Yoder et al., 2006; [31]MacMillan et al., 2009.

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

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

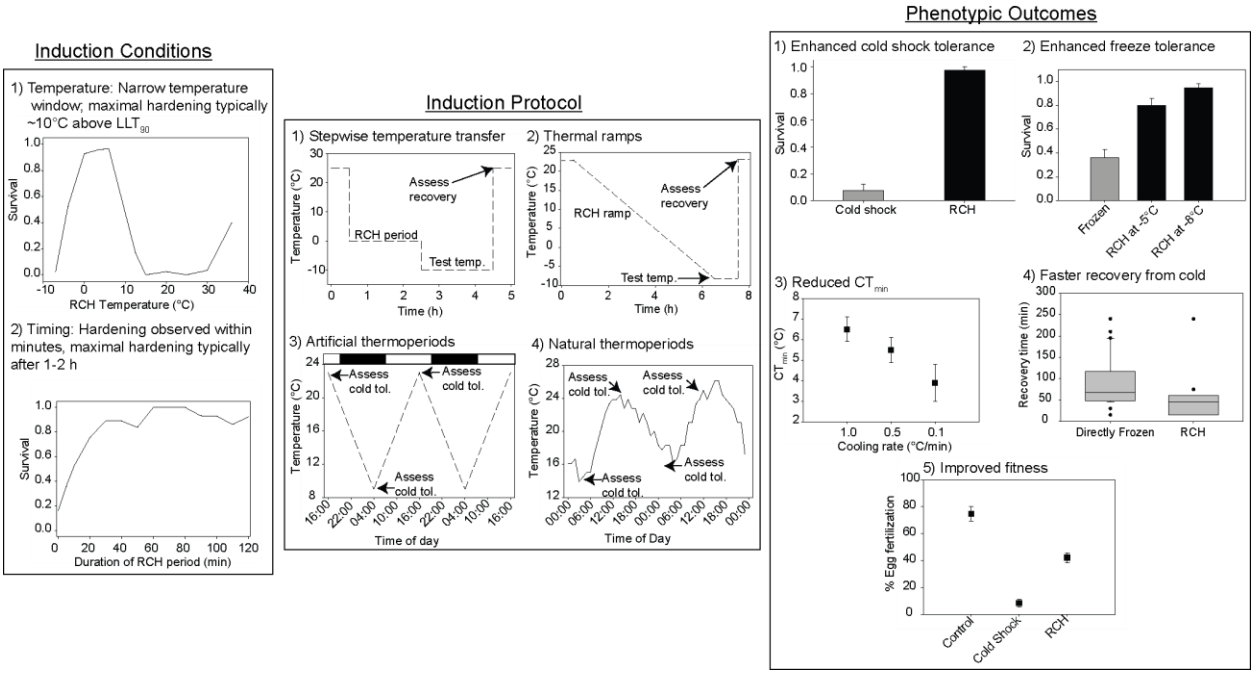
Tables

Table 1. Summary of triggers that elicit rapid acclimation within 24 h and their effects on stress tolerance.

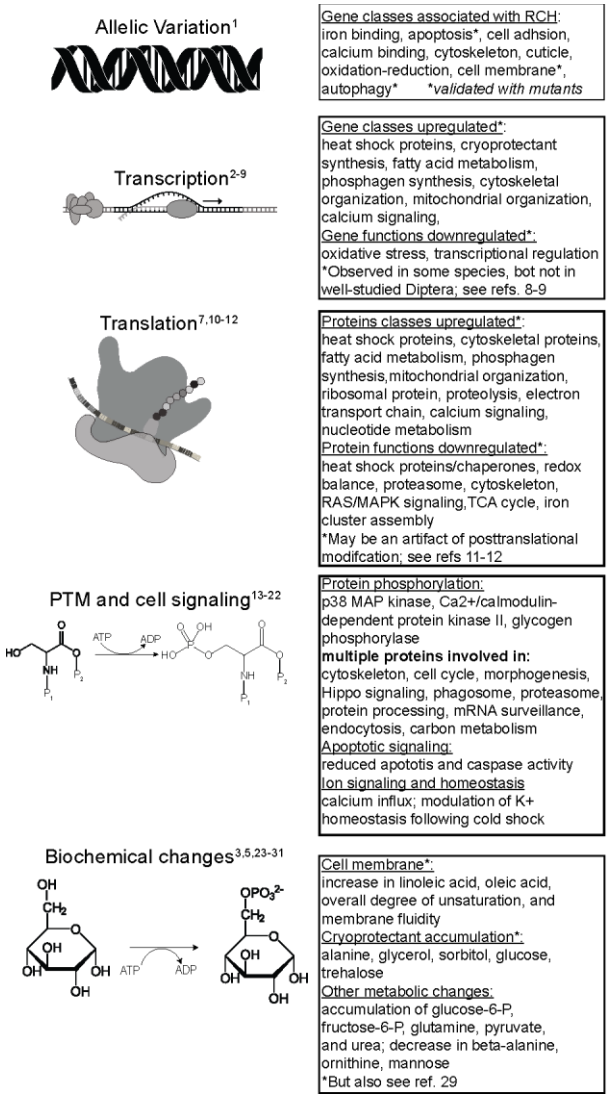
Pretreatment condition (in **bold**) and effects	Selected references
Chilling	
↑ cold tolerance	Lee et al., 1987 and numerous others
↑ freezing tolerance	Kawarasaki et al., 2013
↑ dehydration resistance	Yoder et al., 2006
↑ anoxia tolerance	Gantz et al., in review
↑ immune system activity	Salehipour-shirazi et al., 2017
Freezing	
↑ freezing tolerance	Lee et al., 2006; 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., in preparation
Hypoosmotic stress	
↑ freezing tolerance	Gantz et al., in preparation
Anoxia	
↑ cold tolerance	Coulson and Bale, 1991
Fasting	
↑ freezing tolerance	Gantz et al., in preparation
UV irradiation	
↑ freezing tolerance	Gantz et al., in preparation

Figures

Figure 1



1059 Figure 2



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