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COMMENTARY

Mechanisms underpinning the beneficial effects of fluctuating thermal regimes in insect cold tolerance

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ABSTRACT

Insects exposed to low temperature often have high mortality or exhibit sublethal effects. A growing number of recent studies have shown beneficial effects of exposing insects to recurrent brief warm pulses during low-temperature stress (fluctuating thermal regime, FTR). The physiological underpinnings of the beneficial effects of FTR on cold survival have been extensively studied over the past few years. Profiling with various '-omics' techniques has provided supporting evidence for different physiological responses between insects exposed to FTR and constant low temperature. Evidence from transcriptomic, metabolomic and lipidomic studies points to a system-wide loss of homeostasis at low temperature that can be counterbalanced by repair mechanisms under FTR. Although there has been considerable progress in understanding the physiological mechanisms underlying the beneficial effects of FTR, here we discuss how many areas still lack clarity, such as the precise role(s) of heat shock proteins, compatible solutes or the identification of regulators and key players involved in the observed homeostatic responses. FTR can be particularly beneficial in applied settings, such as for model insects used in research, integrated pest management and pollination services. We also explain how the application of FTR techniques in large-scale facilities may require overcoming some logistical and technical constraints. FTR definitively enhances survival at low temperature in insects, but before it can be widely used, we suggest that the possible fitness and energy costs of FTR must be explored more thoroughly. Although FTR is not ecologically relevant, similar processes may operate in settings where temperatures fluctuate naturally.

KEY WORDS: Cold storage, Physiological repair, Fluctuating temperature, Recovery

Introduction

Because of thermodynamic effects on biochemical processes, temperature determines physiological functions, which underlie development, fitness and performance (Hochachka and Somero, 2002). This is particularly important for ectotherms, for which body temperature is determined by environmental temperature. As temperature declines, insects lose membrane potential of nerves and muscles, causing a loss of exitability, which causes entry into chill coma (Andersen et al., 2018). Although most insects have the capacity to recover from a brief chill coma, prolonged periods of chilling may result in cumulative complex physiological damage that can severely compromise their survival (Chown and Nicolson, 2004).

The ability of insects to survive cold has been well studied (e.g. Chown and Nicolson, 2004; Lee, 2010). Generally, these studies have been conducted under constant low temperature (CLT) (Colinet et al., 2015). The reason that so much research has been done in this area is the drive to understand winter survival, which for many species involves prolonged exposure to cold. Protocols using CLT have likely received more attention than protocols with fluctuating temperature because of their greater simplicity, the need for standardized comparative approaches and the compelling attraction towards the 'golden mean'. However, the natural thermal environment is hardly ever stable but, rather, fluctuates on scales ranging from hours to seasons (Helmuth et al., 2010).

In their natural environment, insects are exposed to daily temperature variations associated with thermal stochasticity. Hence, species must be pre-adapted to frequent and unpredictable thermal variations. Insects must also be able to quickly recover from chill injuries that may accumulate during unpredictable cold periods. Based on these notions, scientists started to explore cold tolerance under fluctuating temperature (FT). Reports from the 1970s first showed that using FT improved the thermal tolerance of insects compared with those exposed to CLT (Casagrande and Haynes, 1976; Meats, 1976). A growing body of literature then supported that the responses of insects to CLT differed markedly from the responses of insects to FT under both field and laboratory settings (Colinet et al., 2015). Over recent decades, interest about the impact of FT (as opposed to constant exposures) has grown in entomological science, due mainly to the increasing awareness of the importance of thermal variance and extremes in the current global changes (Helmuth et al., 2010; Colinet et al., 2015; Dillon et al., 2016).

Fluctuating temperature is a generic term that refers to any discontinuous thermal regime that occurs on a short-term basis. There is an increasing concern about the methodological context of laboratory studies, especially regarding the transferability of results based on constant temperatures to more realistic field situations. Consequently, a large number of FT studies were designed to better appreciate ecologically relevant microhabitat variability and incorporate this into lab-based experiments (Dillon et al., 2016). In contrast, many other FT studies were designed with an applied perspective: using FT to extend cold survival in the laboratory. In this Commentary, we focus on these latter studies where prolonged cold exposure is interrupted by artificial warming breaks (single or repeated), referred to as a fluctuating thermal regime (FTR). We exclude repeated cold exposure (RCE) studies that incorporate repeated short cold shocks (comprehensively reviewed by Marshall and Sinclair, 2012). Understanding how FTR (i.e. short breaks in chronic cold exposure) promotes cold tolerance of insects is fascinating, and the promise of its use in improving insect cold tolerance is exciting. Despite the fact that

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FTR is not an ecologically relevant treatment, it remains conceivable that similar processes may operate in settings where temperatures fluctuate naturally.

Beneficial effects of FTR

Exposing insects to FTR versus CLT significantly reduces cold-induced mortality in most species tested to date (Table 1). Since the pioneering experiments that incorporated a regular recurring high-temperature pulse during cold exposure (Pullin and Bale, 1989; Leopold, 1998; Nedvěd et al., 1998), the benefits of FTR have been clear: insects exhibit increased survival under FTR compared with CLT. Subsequently, multiple studies have shown the benefits of FTR in a steadily increasing number of species from a variety of taxa. Currently, insect species from six different orders (Table 1) have exhibited increased longevity under FTR. Importantly, this is not limited to naturally cold-hardy species, as benefits of FTR for both temperate and tropical species have been reported. Nor is it limited to a physiological state that is normally stress tolerant (i.e. during diapause). While many studies have shown FTR to be effective for quiescent insects, others have shown efficacy on developing insects as well (e.g. Boardman et al., 2013; Rinehart et al., 2013; Koštál et al., 2016). The response is also not limited to specific life stages, as larval, pupal and adult insects all exhibit improved cold tolerance in response to FTR as compared with their counterparts exposed to CLT (Table 1).

Two hypotheses could explain the benefits of FTR. First, fewer chill injuries may accumulate from day to day, because insects are exposed to low temperatures for a shorter cumulative period of time ('lower cold dose' hypothesis). Alternatively, the effects of chilling may be periodically repaired during warming intervals ('physiological recovery' hypothesis). Evidence does not support the lower cold dose hypothesis, because the cold survival remains much higher under FTR than under CLT, even when comparing strictly equivalent doses of low temperature (e.g. Hanč and Nedvěd, 1999; Renault et al., 2004; Lalouette et al., 2007; Boardman et al., 2013). The evidence in support of the physiological recovery hypothesis is reviewed in the next section.

Mechanisms of FTR

The physiological recovery hypothesis, the generally accepted explanation for the beneficial effect of FTR on survival, states that insects profit from periodic opportunities to physiologically recover from the chilling injuries that accumulate during the cold periods (Hanč and Nedvěd, 1999; Koštál et al., 2007). Therefore, unlike cold acclimation, which protects against future injuries, FTR entails repairing damage that has already occurred (e.g. Nedvěd et al., 1998; Renault et al., 2004; Colinet et al., 2006; Koštál et al., 2007; Torson et al., 2015). However, direct evidence in support of the physiological recovery hypothesis is still scarce; results are generally correlative and can vary among and even within studies (e.g. Dollo et al., 2010; Colinet, 2011; van Dooremalen et al., 2011). In spite of this, a tentative general picture of repair mechanisms under FTR can be painted from the available literature (Fig. 1).

Any abiotic stress, like low temperature, can alter macromolecular structures, such as membranes (lipids), DNA (nucleotides), proteins (structural or enzymes) and filaments (cytoskeleton), as well as their function(s) (e.g. reduced enzyme activity or altered membrane viscosity). These alterations can in turn result in downstream effects on pathways responsible for complex biological processes such as respiration, energy production and general homeostasis (Korsloot et al., 2004; Kültz, 2005). Repair mechanisms during FTR are presumably acting on all these targets.

Repair related to effects at the macromolecular level

Restoration of cell membrane structures (phospholipids)

Membranes are thermally sensitive structures and a potential target of chill and freeze injuries (Cao-Hoang et al., 2010). When cooled, the phospholipid bilayer may undergo a transition to a gel-phase state. A high level of membrane permeabilization, resulting from phase separation, can induce a sharp decline in membrane function (Cossins, 1994; Hazel and Hazel, 1995). In addition, loss of active transport across membranes can severely compromise ion and water homeostasis (Overgaard and MacMillan, 2017). Recent data in *Drosophila* reveal strong alterations in the composition of membrane phospholipids under CLT (Colinet et al., 2016). By contrast, during the warming intervals of FTR, the phospholipid composition was repeatedly regenerated (Colinet et al., 2016), likely contributing to the re-establishment of membrane function and that of its associated proteins (e.g. ion pumps). The fatty acid composition of the springtail *Orchesella cinctawas* was also affected by FTR, but a direct link with cold tolerance could not be established (van Dooremalen et al., 2011). Indeed, fatty acid composition varied with the first temperature cycles, then stabilized and resembled that found under warm temperature conditions (van Dooremalen et al., 2011). Along the same lines, transcriptomic data comparing CLT- and FTR-exposed bees showed differential expression of transcripts related to the structural components of membranes (e.g. aquaporins and transmembrane proteins), as well as increased expression of desaturases (i.e. enzymes capable of catalyzing the desaturation of fatty acids), indicating that the CLT- and FTR-exposed bees may have distinct membrane structure and functionality (Torson et al., 2017). Together, these data suggest that one of the mechanisms of FTR is to repair putative cold-induced membrane alterations or to change membrane structure to optimise and maintain functions at low temperature. Membrane damage/repair could be tested using dye exclusion/entry into cells, assessment of biomarkers leaking out of cells or changes in membrane biophysical conditions.

Maintenance of proteostasis using molecular chaperones

Protein unfolding is another direct effect of stress, and protein structure is affected at both extremes of the temperature spectrum (Privalov, 1990; Tsai et al., 2002). Inhibition of proteins due to conformational changes results in many kinds of effects within cells, such as impairment of enzymatic functions and metabolic processes (Korsloot et al., 2004). Molecular chaperones such as heat shock proteins (HSPs) typically accumulate during the warming period following cold stress (Yocum, 2001; Koštál and Tollarová-Borovanská, 2009; Colinet et al., 2010a). HSPs contribute to cold tolerance and help to repair chill injuries (Koštál and Tollarová-Borovanská, 2009; Rinehart et al., 2007; Colinet et al., 2010b), most likely through refolding of partially denatured proteins. Several studies have detected HSP expression during warm periods of FTR. A proteomic study in parasitic wasps, *Aphidius colemani*, found that HSP70 and HSP90 accumulate under FTR (Colinet et al., 2007a). In *Pyrrhocoris apterus*, the level of *hsp70* mRNA increased more than 1000-fold during warming periods (Tollarova-Borovanska et al., 2009), and *Thaumatotibia leucotreta* exposed to FTR also had higher levels of HSP70 (Boardman et al., 2013). In all these cases, cold survival was notably promoted by FTR, and a higher abundance of molecular chaperones was repeatedly detected at the transcript and/or protein level. Mobilization of HSPs is therefore a prime candidate mechanism for repair under FTR, if the recovery duration is long enough to allow protein synthesis. While it is established that HSPs play roles in cold tolerance (Koštál and Tollarová-Borovanská, 2009;

Table 1. An overview of fluctuating thermal regime (FTR) studies that investigated phenotypic and/or physiological effects of FTR in insects and Collembola

Order	Species	Life stage[a]	FTR temperature (°C)[b]	FTR cycle, frequency	CLT (°C)[c]	Phenotypical response[d]	Physiological measures	Reference
Collembola	*Orchesella cincta*	A	–3/5 to 30	20–23.5/ 4–0.5 h, daily or every 2 or 3 days	–5 to 1	↑ Survival		Nedvěd et al. (1998)
			5/50	2/2 days	5		Fatty acid composition, thermal tolerance	van Dooremalen et al. (2011)
Coleoptera	*Alphitobius diaperinus*	A	0/20	12/12 h, daily	0	↑ Survival	Amino acids, sugars, polyols	Lalouette et al. (2007)
		A	0/20 or 5/20	12/12 h or 4/20 h, daily	0 or 5	↑ Survival	Respirometry, oxidative damage	Lalouette et al. (2011)
	Smicronyx fulvus	L	6/18 or 6/20	12/12 h or 3/21 h, daily	6	↑ Emergence		Prasifka et al. (2015)
	Alphitobius diaperinus	A	5 or 0/ 10 to 30	22/2 h, daily	0 or 5	↑ Survival		Renault et al. (2004)
		A	6 or 12/25	22/2 h, daily	6 or 12	↑ Fecundity		Renault (2011)
		A	0 to 15/ 5 to 20	23.5 to 20/ 0.5 to 4 h, daily	0–15	↑ Survival		Colinet et al. (2011)
		A	3/20	22/2 h, daily	3	↑ Survival	Adenosine triphosphate level	Colinet (2011)
	Merizodus soledadinus	A	–6 or –4/ 0–12	22/2 h, daily	–6 or –4	↑ Survival		Lalouette et al. (2012)
Diptera	*Eurosta solidaginis*	L	0/20	4/3 days	NA		Cryoprotectant (sorbitol, glycerol)	Pio and Baust (1988)
	Bactrocera latifrons	A	5 or 8 or 11/20	22/2 h, daily	5 or 8 or 11	↑ Survival, ↓fertility		Takano (2014)
	Sarcophaga crassipalpis	ph-A	0/15	6 h warm interruption, once	0	↑ Emergence		Chen and Denlinger (1992)
	Drosophila melanogaster	A	5/20	22/2 h, daily	5	↑ Survival	Metabolic and phospholipid profiling	Colinet et al. (2016)
	Sarcophaga crassipalpis	ph-A	0/15 or 20	24 h warm interruption, once	0	↑ Emergence	Adenosine triphosphate level	Dollo et al. (2010)
	Drosophila melanogaster	A, P	2 to 5/20	22/2 h, daily	2 or 5	↑ Survival		Javal et al. (2016)
		L	5 or 6/11	20/4 h, daily	5 or 6	↑ Survival	Metabolomics, transcriptomics, ion, oxidative stress, biochemical composition, adenosine triphosphate level	Koštál et al. (2016)
	Musca domestica, *Lucilia cuprina*, *Lucia sericata*	P	10/28	23 or 47/1 h or 93/3 h or 94/2 h	10	↑ Emergence		Leopold et al. (1998)
Hemiptera	*Pyrrhocoris apterus*	A	–5/25	22/2 h, daily	–5	↑ Survival	Heat shock protein 70	Tollarova-Borovanska et al. (2009)
	Pyrrhocoris aperus, *Alphitobius diaperinus*	A	–5/20 or 4/25	22/2 h, daily	–5 or 4	↑ Survival	Metal ion concentration, mass, hydration	Koštál et al. (2007)
	Pyrrhocoris apterus, *Orchesella cincta*	L	–5/0 to 35	23.5 to 20/ 0.5 to 4 h, daily	–5	↑ Survival		Hanč and Nedvěd (1999)

Continued

Table 1. Continued

Order	Species	Life stage[a]	FTR temperature (°C)[b]	FTR cycle, frequency	CLT (°C)[c]	Phenotypical response[d]	Physiological measures	Reference
Hymenoptera	*Aphidius colemani*	P	4/20	22/2 h, daily	4	↑ Emergence, ↑male fitness, ↑locomotion		Colinet and Hance (2009)
	Ephedrus cerasicola, *Praon volucre*, *Aphidius matricariae*, *Apidius ervi*	P	2/20	22/2 h, daily	2	↑ Emergence, ↓Development time		Colinet and Hance (2010)
	Aphidius colemani	P	4/20	22/2 h, daily or every 2–3 days	4	↑ Emergence		Colinet et al. (2006)
		P	4/20	22/2 h, daily	4	↑ Emergence	Free amino acids	Colinet et al. (2007b)
		P	4/20	22/2 h, daily	4	↑ Emergence	Proteomics	Colinet et al. (2007a)
	Aphidius ervi	P	7/20	22/2 h, daily	7	↑ Emergence, ↓Development time, longevity, fecundity	Mass, total fat, water	Ismail et al. (2010)
		P	0 or 4/20	22/2 h, daily	0 or 4	↑ Emergence, ↓development time, ↓longevity, fecundity	Total fat	Ismail et al. (2013)
	Lysiphlebus fabarum	L,P	6 or 8/21	22/2 h, daily	6 or 8	Emergence, development time, body size, egg load		Mahi et al. (2014)
	Diadromus pulchellus	A	4/13.5	1 or 3 warm pulses	4	Survival, fecundity, sex ratio		Murdoch et al. (2013)
	Megachile rotundata	P	6/20	23/1 h, daily	6	↑ Emergence		Rinehart et al. (2011)
		P	6/20	23/1 h, daily	6	↑ Emergence, longevity		Rinehart et al. (2013)
		P	6/15 to 25	23 to 21/ 1 to 3 h, daily	6	↑ Survival		Rinehart et al. 2016
		P	6/20	23/1 h, daily	6	↑ Survival	Transcriptomics	Torson et al. (2015)
		P	6/20	23/1 h, daily	6	↑ Survival	Transcriptomics	Torson et al. (2017)
		P	6/15 or 20	23 or 22/ 1 or 2 h, daily	6		Respirometry	Yocum et al. (2011)
		P	6/20	5–120 min daily or every 1/2, 1, 2, 7 days	6	↑ Emergence, ↓time		Yocum et al. (2012)
Lepidoptera	*Mamestra configurata*	P	−14.5/20	1–48 h warm interruption, once	−14.5	↑ Emergence		Turnock and Bodnaryk (1993)
	Thaumatotibia leucotreta	L	−5/20	30 min hold at each temperature	−5	↑ Survival, ↑Pupation rate	Mass, water, protein, heat shock protein 70, metabolic rate	Boardman et al. (2013)
	Aglais urticae and *Inachis io*	A	−5/10	16/8 h, 5 days/ week	−5	↑ Survival	Weight loss, supercooling point	Pullin and Bale (1989)
Orthoptera	*Locusta migratoria*	E	−10/5 to 35	23.5–20/ 0.5–4 h, daily	−10	↑ Hatching	Oxidative defense and ion pump activity under CLT	Jing et al. (2005)

[a]Life stage: E, egg; L, larvae (or nymph); P, pupae (or pre-pupae); A, adult; ph-A, pharate adult.
[b]Upper/lower values for low/high temperatures used in the FTR cycles.
[c]Constant low temperature (CLT) conditions that FTR was compared to (NA, not compared).
[d]Arrows indicate the direction of the phenotypic change with respect to conditions of CLT. The absence of an arrow means no change compared with CLT.

Rinehart et al., 2007; Colinet et al., 2010b), direct evidence of their role(s) in FTR-related repairs is lacking. This could be tested via genetic tools (e.g. RNAi, fly mutants), which would allow testing of whether abrogating the expression of specific HSP mRNAs might be associated with a lowering of the survival advantage that would otherwise by gained by FTR.

Cytoskeleton and DNA integrity

Cytoskeletal instability/disassembly is another direct consequence of cold stress (Cottam et al., 2006; Kim et al., 2006; Des Marteaux et al., 2018). Both actin and microtubules participate in nuclear positioning during the cell cycle, and both affect chromatin and DNA behavior (Andrin et al., 2012; Lawrimore et al., 2017). Because the cytoskeleton is tightly linked to cell membranes, disruption of cytoskeletal elements is also associated with membrane dysfunction (Lázaro-Diéguez and Egea, 2007). A proteomic study found that actin depolymerizing factor, which regulates actin polymerization, was up-regulated under FTR (Colinet et al., 2007a). Transcripts coding for structural constituents of the cytoskeleton were also expressed during FTR

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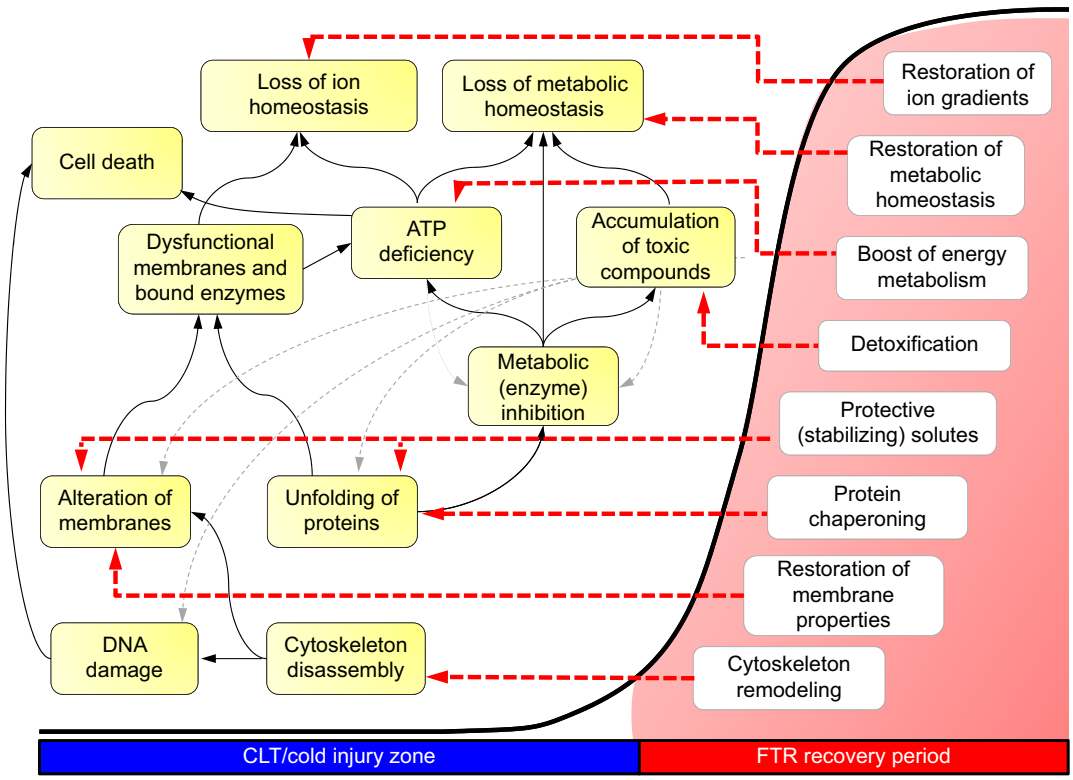

Fig. 1. Summary of the major sources of chill injury and the repair mechanisms occurring under the fluctuating thermal regime (FTR). The back curve represents the increase in temperature during the recovery period of FTR. Within the constant low temperature (CLT)/cold injury zone (indicated by the blue bar), the yellow boxes indicate the main targets of cold stress acting on macromolecular structures, and the downstream consequences on pathways and complex biological processes. Within the recovery period of FTR (shaded red), the white boxes denote repair/restoration mechanisms acting on the multiple alterations resulting from cold stress (indicated by dashed red arrows). Note that repair processes are not necessarily listed in the order that they respond to temperature change. Thin black arrows represent upstream effects of cold stress leading to downstream consequences. Dashed gray arrows indicate potential targets of accumulated toxic compounds.

(e.g. actin, dynein, myosin, myofilin) (Torson et al., 2015, 2017). Considering the relevance of cytoskeletal components in cold stress and its tolerance, it is reasonable to speculate that FTR may help in resetting cytoskeleton integrity. Evidence of this process is not yet available, although work is underway to address this gap.

In addition to the alteration of nuclear and cell membranes, DNA integrity may also be directly compromised by chilling, even at non-freezing temperatures. Chilling has been shown to cause nuclear anomalies (e.g. micronucleus) and chromosomal aberrations (e.g. stickiness, fragmentation or constrictions) (Mishra and Tewari, 2014). Upregulation of several transcripts in cold-stressed (CLT) bees (e.g. *myofilin isoform b*, *sestrin-like* and *DNA damage-binding protein 1*) suggests that individuals may experience DNA damage, potentially caused by increased levels of reactive oxygen species (ROS). This was not observed in FTR-exposed insects (Torson et al., 2017). Whether FTR allows cold-induced genotoxicity to be repaired needs confirmation.

Repair related to downstream effects of stress

Ion homeostasis

A downstream consequence of the putative cold-induced alteration of membranes and a decline in the activity of membrane-bound enzymes (Koštál, 2010; MacMillan et al., 2017) is the progressive loss of ion and water homeostasis. Disruptions in ion and water homeostasis cause neuromuscular alterations, chill coma, chill injury and ultimately death (reviewed by Overgaard and MacMillan, 2017). Under FTR, primary ion-pumping systems were able to re-establish ion gradients across cell membranes during the warm spells in *P. apterus* and *Alphitobius diaperinus* (Koštál et al., 2007). Likewise, quiescent *Drosophila melanogaster* larvae maintain lower concentrations of potassium when exposed to FTR versus CLT (Koštál et al., 2016). RNAseq analysis of the alfalfa leafcutting bee, *Megachile rotundata*, demonstrated an increased abundance of transcripts involved in counteracting disruption of ion homeostasis under FTR (Torson et al., 2015). Together, these results strongly support that re-establishment of ion homeostasis under FTR contributes to repair of chill injury.

Metabolic homeostasis and mobilization of compatible solutes

Deviation of metabolic homeostasis is another symptom of cold stress, likely reflecting the downstream consequences of complex metabolic alterations and damage inflicted on macromolecules (Colinet et al., 2012; Teets et al., 2012; Williams et al., 2014). Investigation of the temporal maintenance/deviation of metabolic networks over the course of FTR revealed that the disturbed metabolic profiles returned towards the initial state by the end of recurrent recovery periods, which means that a fast homeostatic regeneration occurs during warm intervals (Colinet et al., 2016). Another study further supported that warm episodes of FTR generally help to re-establish homeostatic conditions (Koštál et al., 2016) – however, the regulators and key players of this complex process are unknown.

Long-term environmental stress can alter general homeostasis and, in reaction, cells activate the cellular homeostasis response in order to restore homeostasis – for instance, via the accumulation of compatible organic osmolytes (Kültz, 2005). Compatible solutes, such as sugars, polyols or free amino acids (FAAs), can stabilize proteins and membranes at low concentrations (Yancey, 2005). Changes in concentrations of compatible solutes have been detected during the warm period of FTR in several cases, although changes were generally of low magnitude, species specific and, consequently, difficult to generalize. Pio and Baust (1988) reported in *Eurosta solidaginis* that periodic variation in glycerol and sorbitol concentrations occurred with FTR, with concentrations of polyols returning to basal levels upon exposure to warm episodes. In *A. diaperinus*, glycerol accumulated under FTR, while glucose level decreased. Alanine and proline accumulated in FTR-exposed beetles compared with counterparts exposed to CLT (Lalouette et al., 2007). However, the FAA pool, which generally increases under CLT, was found to drop during warm intervals under FTR (Lalouette et al., 2007; Colinet et al., 2007b). Reduction of the total FAA pool under FTR may relate to utilization of amino acids for protein synthesis and energetic processes. Metabolomic studies also detected changes in levels of certain metabolites with cryoprotective functions under FTR, such as FAAs (proline, alanine) and sugars (fructose) (Colinet et al., 2016; Koštál et al., 2016). Nevertheless, the concentrations and the magnitude of the fold-changes were too low to allow speculation about colligative function. While these data point to differential use of compatible solutes between insects exposed to FTR and CLT, these patterns are only correlative, and the precise role(s) of these molecules as drivers of increased tolerance under FTR remains to be verified, using, for instance, ^{13}C-labeled metabolites or genetic manipulation targeting the biosynthesis of candidate metabolites.

Boost of respiration and energy metabolism

Low temperature compromises mitochondrial ATP production (Colinet et al., 2017). It has been suggested that energy supply may be regenerated by the warming pulses under FTR (Chen and Denlinger, 1992). The up-regulation of energy production pathways during the warm recovery period is certainly important to provide the fuel for energy-demanding repair mechanisms. Several studies have reported that insects resume very active respiration during warming spells of FTR, manifested as overshoots in metabolic rate (Lalouette et al., 2011; Yocum et al., 2011; Boardman et al., 2013). Proteomic work also points to up-regulation of energy production pathways during warming intervals (Colinet et al., 2007a). Metabolomics studies suggest that intermediate metabolism, including glycolysis and tricarboxylic acid (TCA) turnover, was slowed down by chilling but this decrease occurred faster under CLT than under FTR (Koštál et al., 2016). In terms of the production of energy equivalents, an increase in ATP level was reported in the flesh fly *Sarcophaga crassipalpis* during a single long warming pulse (24 h) (Dollo et al., 2010), but this was not observed in *A. diaperinus* exposed to recurrent short warming pulses (2 h) (Colinet, 2011). Together, these data attest that an active regulation of energy metabolism and metabolic rate occurs during warming spells, most likely to support repair mechanisms.

Protection from ROS

Insects exposed to CLT may experience elevated levels of ROS (Joanisse and Storey, 1998; Jing et al., 2005), and a periodic increase in temperature could alleviate the resulting oxidative stress via up-regulation of antioxidant and detoxification systems. In *A. diaperinus*, cold stress provoked oxidative damage, and the warm periods under FTR activated the antioxidant system (Lalouette et al., 2011). A large number of transcripts involved in the oxidative stress response, including transcripts of genes encoding *peroxidase*, *GST* and several *CYP450* proteins, were up-regulated under FTR in a solitary bee (Torson et al., 2015). Levels of lipid hydroxyperoxides and protein carbonyls were assessed in *D. melanogaster* fly larvae exposed to CLT and FTR, and both oxidative damage biomarkers were increased under CLT, which suggests that the antioxidant system operates more efficiently under FTR (Koštál et al., 2016). These observations support the view that FTR acts to reduce oxidative stress.

Practical applications of FTR

Cold storage is commonly used in mass rearing of insects to increase the duration for which they can be successfully stored (referred to in the industry as 'insect shelf-life'), improve synchronization for field releases and increase overall operational efficiency. This is true not only for integrated pest management and biocontrol programs (Leopold, 1998) but also for other industries such as those associated with entomophagy, pollination services or the preservation of threatened species (Leopold, 2007). Given the beneficial effects of FTR, the potential for its use in applied situations is clear, although it remains under-utilized. Additionally, the simplicity and seemingly ubiquitous nature of the FTR response make it especially useful in an applied setting owing to the complexities associated with other storage techniques. For instance, using diapause induction for storage requires knowledge of the species' physiology (Denlinger, 2008) and cryopreservation requires extensive protocol development for each species (Leopold and Rinehart, 2010).

The use of FTR to assist biocontrol programs has been extensively studied. For instance, the shelf-life during short-term cold storage (i.e. for a few weeks) of several species of parasitic wasps, especially those that attack aphids, has been shown to be markedly improved by employing FTR (Colinet and Hance, 2009, 2010; Ismail et al., 2013; Mahi et al., 2014), which could allow much greater flexibility in the rearing and distribution of these biocontrol agents. Sterile Insect Technique (SIT) programs could also benefit from adopting FTR as part of their operating procedures. Successful SIT programs require sterile insects to be stored in the cold without loss of performance and to be quickly mobilized. Sterile insects are also exposed to low temperature, sometimes for several days, during shipping from mass-rearing facilities to release sites (Nikolouli et al., 2017). The addition of FTR may be useful in improving the survival of insects during low-temperature exposures used in such programs.

FTR can also be used to improve pollination services, as has been demonstrated in the alfalfa leafcutting bee, *M. rotundata*. While this intensively managed solitary bee can be used to pollinate a variety of crops (Pitts-Singer and Cane, 2011), its month-long spring developmental period must be closely synchronized with crop bloom, a process that can be substantially complicated by weather abnormalities (Bosch and Kemp, 2005). Cold storage using CLT during spring development has long been used to delay emergence of adult *M. rotundata* (Undurraga and Stephen, 1980; Yocum et al., 2010), but employing FTR during this cold storage period significantly increases the length of time that *M. rotundata* can be stored to delay development (Rinehart et al., 2011, 2016) and increases the longevity of the resultant adults in comparison with

those stored under CLT (Rinehart et al., 2011; Bennett et al., 2015). In addition, FTR can be used to improve long-term storage of this pollinator species. When stored during the winter months as diapausing and post-diapause quiescent prepupae, survival is substantially improved by use of a daily warm period, with few deleterious effects (Rinehart et al., 2013; Bennett et al., 2013). In fact, storing bees under FTR increased their shelf-life from approximately 9 months to up to 20 months without increased mortality, essentially extending their usability for pollination managers for an additional field season. This increased storage time could not only help protect the industry from yearly supply fluctuations but also be used as a means of germplasm storage, allowing managers to propagate bees every other year.

To date, the largest use of FTR as a practical tool is in the research setting. Protocols for the red sunflower seed weevil, *Smicronyx fulvus*, were specifically designed to reduce the seasonality of research on this species and to protect against the unpredictability of natural populations (Prasifka et al., 2015). An additional benefit for this species is that incubation under FTR also increases the speed of diapause progression, making developing individuals available for research purposes earlier in the season. The use of FTR during diapause and post-diapause quiescence in *M. rotundata* is a well-documented method for extending seasonal research as well, although the effects of aging on the parameter being investigated should first be tested (Yocum et al., 2016). The greatest impact of FTR in the research setting may be its use in sustaining scientific collections, especially for the maintenance of insect stock centers. For instance, *D. melanogaster* responds well to FTR (Javal et al., 2016; Koštál et al., 2016), and a report by the National Institutes of Health indicates that FTR should be a component of a comprehensive strategy of *Drosophila* stock center maintenance (https://orip.nih.gov/sites/default/files/Cryopres%20workshop%20report%20final%2012-28-16.pdf). Other stock centers, such as the Malaria Research and Reference Reagent Resource Center (MR4), could also profit from FTR protocols to reduce maintenance costs.

Downsides to FTR

Although FTR seems to be one of the silver bullets for surviving cold stress, there are possible fitness and energy costs for insects. One might expect a tradeoff when testing the insect response to FTR, i.e. increased survival of cold may come at a cost to fitness. Although the warming period is thought to allow repair processes to function, this also requires energy, which would normally be allocated to reproduction. There is some evidence for this in *Bactrocera latifrons* (Takano, 2014), where FTR treatment reduced the number of insects laying viable eggs. Fecundity was also reduced after fluctuating treatment in *Zeiraphera canadiensis* (Carroll and Quiring, 1993), *D. melanogaster* (Marshall and Sinclair, 2010) and *Ceratitis capitata* (Basson et al., 2012). In another case, FTR slightly reduced adult longevity (Ismail et al., 2013), which could reduce lifetime fecundity if that species continues to reproduce throughout the adult stage. However, in another study using *Diadromus pulchellus*, there was no effect of FTR on the number of mated females or fecundity compared with CLT (Murdoch et al., 2013). The rationale for the existence of a fitness cost is that, during recurrent warm periods of FTR, the overshoot in metabolic rate (Lalouette et al., 2011; Yocum et al., 2011; Boardman et al., 2013) may progressively consume more energy stores than under CLT. Indeed, in a few cases, the fat content of insects treated with FTR was lower than that of control animals (Ismail et al., 2013), which may have accounted for the decrease in adult longevity seen in those insects. *Aglais urticae* and *Inachis io* butterflies exposed to FTR lost more weight than those exposed to CLT (Pullin and Bale, 1989). However, other cases found no effect on energy stores, with fat content being the same as or greater than that of CLT-exposed insects (Ismail et al., 2010). Clearly, there is a need to assess whether increased survival resulting from FTR entails fitness consequences.

While a few studies have looked at the reproductive costs of FTR in females (e.g. Carroll and Quiring, 1993; Marshall and Sinclair, 2010; Ismail et al., 2013), consequences for males are much less well known. A study on *A. colemani* found that cold survival as well as parameters related to male reproductive potential were negatively affected in wasps under CLT – by contrast, insects exposed to FTR were not affected. Alterations of reproductive potential in CLT-exposed males were mainly associated with decreased locomotion performance (Colinet and Hance, 2009). Because prolonged CLT results in the loss of ion balance, alterations of muscular resting potentials and neuronal conduction (Kelty et al., 1996), it is not surprising that CLT-exposed insects may suffer from motility defects, such as uncoordinated movements (Koštál et al., 2006). Because FTR allows ion homeostasis to be maintained (Koštál et al., 2007, 2016), negative effects of chilling on neuromuscular function can certainly be mitigated, which should preserve locomotor performance. Clearly, the energetic and fitness costs of FTR have not been sufficiently explored and further efforts should be undertaken to fill this knowledge gap.

Confounding effects

From an applied point of view, FTR protocols are indubitably useful to prolong the shelf-life of important insects in small-scale facilities. Yet, its application in industrial insect production may face technical difficulties. While it is relatively easy to quickly shift the temperature of small-volume cabinets within a short time, recurrent, brief shifts in temperature of large-scale rearing units (e.g. room sized) may be much more technically challenging and energy consuming. Furthermore, shifting the temperature also directly affects relative humidity (RH). When temperature decreases after warm episodes without changing the moisture content of the air, RH will eventually reach 100%, and water vapor will start to form condensation. Changes in RH are well known to affect insect physiology (e.g. Boardman et al., 2013), and variation of RH that accompanies FTR may directly affect insects' cold survival as well. Indeed, high humidity can promote cold survival of *Drosophila* at some temperatures (Kobey and Montooth, 2013), but this is not always true (Boardman et al., 2013; Enriquez and Colinet, 2017). FTR experiments should be explored at both constant and variable levels of RH to disentangle the effects of each. The free water that may condense on the surfaces of rearing containers under FTR, associated with warm intervals, may also create environments that are highly conducive to microbial and fungal growth (Sikorowski and Lawrence, 1994). Whether FTR favors contamination of insect colonies and the possible cost of immune responses have yet to be evaluated. Lastly, when feeding stages are stored under FTR with food supply, insects may ingest food during warm episodes, whereas insects stored at CLT are starving the entire time (Koštál et al., 2016). Therefore, positive effects of FTR in some cases may result from factors other than temperature per se.

Conclusions and perspectives

Evidence from multiple studies points to direct effects of stress on molecular structures, eventually leading to the loss of general homeostasis at CLT. In contrast, FTR allows repair mechanisms to

function and the re-establishment of physiological homeostasis (e.g. ion balance, metabolism, membrane function). Although considerable progress has been achieved in understanding the physiological mechanisms underlying the beneficial effects of FTR, there are still many areas that lack clarity, such as the precise role(s) of HSPs and compatible solutes or the identification of regulators and key players involved in the observed homeostatic responses. For these reasons, studies on FTR keep opening exciting new avenues for exploration and basic research.

From an applied point of view, the purpose is to extend the shelf-life regardless of the mechanism. However, from a fundamental point of view, a global understanding of FTR requires disentangling thermal effects from confounding effects, such as fluctuations of RH and re-nutrition. Understanding the mechanism by which FTR improves survival at low temperatures will further lead to the ability to predictably manipulate the FTR technique and thereby maximize the use of this technique across species.

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Competing interests
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