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Current Opinion in Insect Science

Molecular mechanisms and trade-offs underlying fluctuating thermal regimes during low-temperature storage

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Insects exposed to constant low temperatures (CLT) exhibit high rates of mortality as well as a variety of sublethal effects. In many species, interruptions of CLT with brief pulses of warm temperatures (fluctuating thermal regimes, FTR) lead to increases in survival and fewer sublethal effects. However, we still lack a complete understanding of the physiological mechanisms activated during FTR. In this review, we discuss recent advances in understanding FTR's underlying molecular mechanisms. We discuss knowledge gaps related to potential trade-offs between FTR's beneficial effects and the costs of these repairs to overwintering reserves and reproduction. We present the hypothesis that the warm pulse of FTR helps to maintain daily rhythmicity.

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Current Opinion in Insect Science 2024, **62**:101160

This review comes from a themed issue on **Molecular physiology**

Edited by **Nicholas Teets** and **Heath MacMillan**

For complete overview about the section, refer "Molecular physiology (2023)"

Available online 11 January 2024

https://doi.org/10.1016/j.cois.2024.101160

Introduction

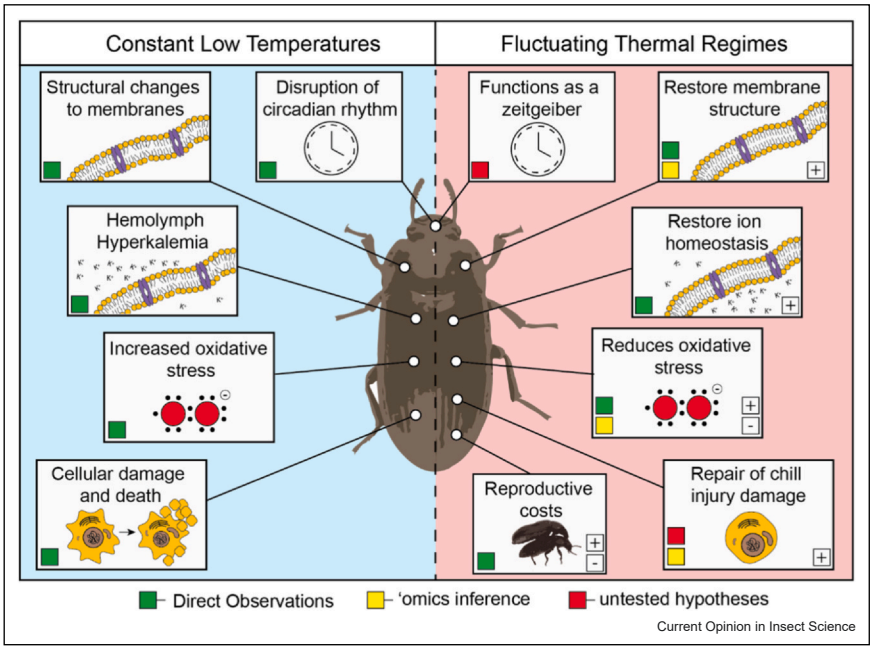
Insect physiology is dynamic and tightly linked with temperature. Through acclimation, most temperate insects can tolerate exposure to stressful low temperatures, whether during temporary cold exposure or through physiological programming associated with diapause. But, extended exposure can lead to an accumulation of cellular damage and neuromuscular dysfunction known as chill injury [1]. Chill injury is particularly problematic in managed insect species that have been stored at constant low temperatures (CLT) for extended periods of time during winter months. CLT is used for storage of biological control agents (e.g. [2]) and agricultural pollinators (e.g. [3]). The chill injury caused by CLT storage has correlates in nature, when insects are exposed to cold snaps during unpredictable weather. Climate change will likely increase the variability of winter temperatures due to snow melt [4], potentially increasing chill injury. Not surprisingly, the details of thermal profiles have ramifications for insect physiology, survival, and lifetime reproductive success. Under CLT, insects exhibit a dramatic reduction in cold-induced mortality if exposed to brief, daily pulses of warm temperatures (fluctuating thermal regimes, FTR).

The benefits of FTR on the survival of chill-injured insects appear to be nearly universal. However, many managed insects are stored in the winter under CLT and have no opportunity to feed or replenish energy reserves [5]. Therefore, it is widely assumed that the energetic costs of cellular repair during FTR will cause a trade-off in other aspects of performance — such as reproductive output (e.g. [6]). In this review, we discuss 1) physiological support for -omics-inferred molecular mechanisms of FTR, 2) potential trade-offs between FTR's benefit and energy allocation, and 3) additional hypotheses that warrant further investigation. Although FTR treatments have been applied in a variety of contexts, the mechanisms of FTR have been most thoroughly studied in insects exposed to CLT. Therefore, for this review, we focus solely on FTR in the context of CLT.

Physiological support for -omics inferences

Improved performance and survival outcomes under FTR have been reported across several insect orders [7]. Broad-scale '-omics' experiments suggest multiple conserved physiological mechanisms, including processes promoting ion, metabolic, and osmotic homeostasis, as well as restructuring of cell membranes, stabilization of the cytoskeleton, increased immune activity and detoxification, and mitigation of oxidative stress (for a detailed review, see Ref. [8]). Direct observations supporting several of these mechanisms are still limited, but recent progress has been made in the context of ion homeostasis [9,10], membrane composition and function [11], and oxidative stress [12], which we discuss in more detail below (Figure 1).

Figure 1



A summary of recent advances and proposed future directions in the physiological mechanisms of FTR. Within each hypothesized response box (white), the green boxes in the lower left-hand corner represent direct observations testing the hypothesis, yellow boxes indicate -omics inferences that support the hypothesis, and red boxes show that the hypothesis has not yet been tested. The + or – in the lower right-hand corner represents whether the direct observations of that response support (+) or do not support (–) the hypothesis. Boxes containing both + and – indicate conflicting results among studies.

Membranes are among the most thermally sensitive macromolecular structures [13]. Extended cold exposure can lead to decreased rates of active transport, which drive disruptions in ion homeostasis and ultimately result in membrane depolarization, loss of muscle function, and cell death [1,14]. El-Saadi et al. [9] and Grumiaux et al. [10] provided strong support for the ability of FTR to mitigate these disruptions in *Drosophila melanogaster* and *D. suzukii*, respectively. In both instances, adult flies exposed to FTR had lower hemolymph [K^+] relative to CLT. These results are consistent with previous observations in the firebug, *Pyrrhocoris apterus*, and the tenebrionid beetle, *Alphitobius diaperinus* [15]. However, it remains unclear whether the warm pulses associated with FTR simply promote changes in the permeability of cell membranes (restoring passive drift of ions down their concentration gradient) or promote an increase in the enzymatic activity or expression of ion transporters. These mechanisms are likely not mutually exclusive, and recent -omics data supports this [16].

The fluidity of the lipids within cell membranes influences the activity of many important membrane enzymes and transmembrane transport processes. Chilling can result in changes to permeability of membranes and reduced activity of membrane-bound enzymes that rapidly lead to the accumulation of chilling injuries and mortality [8]. In *D. melanogaster* adults, CLT leads to a shift in membrane composition and shorter fatty acyl chains [11]. These changes in composition and structure likely help to maintain membrane fluidity at lower temperatures, but could also alter the membranes' permeability and the function of ion channels [17]. Under FTR, these flies reorganize their membranes to more closely resemble flies that had not experienced cold stress [11]. In addition to membrane reorganization, FTR-exposed insects also increased the abundance of transcripts encoding structural components of membranes (e.g. transmembrane proteins and aquaporins, [16]). Further, Melicher et al. [18] showed that *Megachile rotundata* pupae increase expression of transcripts encoding ion channels after just one day of FTR exposure. Since the regulation of ion and osmotic homeostasis in insects is a complex interaction between gut epithelia and the Malpighian tubules [19], we argue that the field must increase research that mechanistically tests tissue-specific regulation of ion homeostasis.

Changes in membrane permeability also affect mitochondrial function during cold stress [20]. A decrease in mitochondrial function during CLT is likely to have a variety of consequential downstream effects, but oxidative stress has received particularly close attention [12,21–26]. Oxidative stress occurs when the production

of reactive oxygen species (ROS) exceeds an organism's ability to clear excess ROS with antioxidants, leading to damage of lipids, proteins, and DNA [27]. ROS-induced damage could be further compounded in chill-injured insects if enzymatic antioxidants are less efficient as temperature decreases, but to our knowledge, this has not been tested. Therefore, a periodic exposure of warmth in FTR should increase the efficiency of those enzymes, allowing cells to clear the ROS that have built up under CLT. In adult *Alphitobius diaperinus*, superoxide dismutase (SOD) activity increases during low-temperature stress and glutathione ratios increase (indicating decreased oxidative stress) during the warm period of FTR [21]. Consistent with these observations, after exposure to CLT, *M. rotundata* prepupae increase expression of transcripts encoding SOD and glutathione peroxidase, relative to FTR [12]. However, counter to predictions, neither CLT- and FTR-exposed prepupae or pupae differed in their total antioxidant capacity or levels of lipid peroxidation (a common proxy for ROS-induced damage). Importantly, this study measured only lipid peroxidation before the point at which mortality between the two treatments began to diverge. It therefore remains possible that 1) differences in antioxidant capacity between CLT and FTR could emerge as mortality begins to increase in CLT and 2) oxidative damage is occurring at the DNA or protein level. These observations in *M. rotundata*, also contrast with elevated levels of lipid peroxidation and protein carbonylation during CLT exposure in *D. melanogaster* larvae, relative to FTR [28]. We assume that this discrepancy between studies is associated with the underlying cold tolerance of the species, but it could also be driven by underlying differences in metabolic rates between the two species during CLT or differences in the duration of the FTR exposure.

Is there a cost to fluctuating thermal regimes?

While the benefits of FTR on insect survival during CLT are well-established [8], the energetic demands and other potential trade-offs are still uncertain (Table 1). The warm pulse of FTR is associated with a brief 'overshoot' in CO_2 production [21,29]. Lalouette et al. [21] hypothesized this overshoot is a result of activation of the reparative functions associated with FTR restoration of ion [9] and metabolic homeostasis [11,28] and repair of stress-induced damage from chill injury [1] could require significant energetic investment. Costly repair mechanisms could explain the overshoot in metabolic rates during the warm pulse of FTR observed by Lalouette et al. [21] and Yocum et al. [29]. Restoring ion gradients is energetically intensive — requiring the availability of ATP to recover homeostasis via metabolically demanding ion transporters [1]. Over longer durations of FTR exposure, we hypothesize that continually reestablishing ion homeostasis could result in a significant energetic demand on the insect. In support of this hypothesis, *Megachile rotundata* prepupae increase the expression of multiple transcripts encoding ATP-dependent ion channels during the warm pulse of FTR [18]. We appreciate that disentangling the energy requirements of each of these mechanisms individually would be a true challenge, especially given that knockdowns of these critical physiological processes would likely result in lethal phenotypes. Tissue-specific inquiries into the dynamics of mitochondrial respiration and ATP synthesis between CLT and FTR (cf. Colinet et al. [30]) could begin to address in what tissues these costs are being incurred.

If the elevated metabolic rates associated with the warm pulse of FTR cause an increase in energetic demands, this cost should be reflected in the energy consumption rates of FTR-exposed insects. However, existing measurements of body composition and ATP levels during FTR do not provide clear support for this relationship. For example, lipid content over 40 days of FTR exposure in *D. suzukii* adults [10] and *Thaumatotibia leucotreta* larvae [7] exposed to short-term FTR (< 1 day) do not differ relative to individuals exposed to CLT. Further, measurements of ATP levels during FTR are inconsistent across studies. Adult *A. diaperinus* during the first three days of CLT or FTR does not differ in ATP levels [31]. This contrasts with higher ATP levels in FTR-exposed *Sarcophaga crassipalpis* pharate adults. Importantly, the temperature profiles of the FTR differ dramatically between these two studies, with *S. crassipalpis* exposed to both longer bouts of low temperatures and warm pulses during the FTR treatment. Perhaps, longer durations of warm pulses during FTR lead to greater energetic demands. Nevertheless, data on the relationship between FTR exposure and energetic demands are still scarce and will likely require measurements of other energy reserves such as glycogen as well as measurements spanning longer durations of FTR exposure.

The ultimate impact of an energetic cost is a decrease in reproduction. The hypothesis that the benefits of FTR come at a cost to investment in reproduction has recently been scrutinized (Table 1). In most of these studies, FTR exposure comes at no cost to reproduction. However, there are contexts in which FTR exposure negatively impacts reproduction [6,32], and others in which there is a benefit to fecundity [32,33]. In *Drosophila suzukii*, FTR exposure as adults greatly reduces mortality, but has no effect on female fecundity or male mating capacity, whereas exposure to FTR as pupae in this species decreases adult female fecundity [6]. The trends presented in these studies support a more complex relationship between FTR exposure and reproductive output, with life stage of exposure, sex, and duration of the treatments emerging as important factors. In some

Table 1

Studies investigating the energetic and reproductive costs associated with FTR exposure.

Order	Species	Life stage	FTR profile	CLT profile	Duration	Results*	Reference
Lepidoptera	*Thaumatotibia leucotreta*	Larvae	-5°C (75 min) 15°C (30 min),	- 5°C,15°C	< 1 day	Cost to pupation rate	Boardman et al. (2013) [7]
Diptera	*Drosophila suzukii*	Adults	3°C for 20 hours, 25°C for 1 hour 20 min	25°C, 3°C	Up to 160 days (in FTR)	No cost to fecundity compared with 25°C. No cost in lipid reserves	Grumiaux et al. (2019) [10]
Diptera	*Drosophila suzukii*	Pupae: adults	Multiple peak temperatures, durations, and frequencies	*Pupae*: 5 or 7°C *Adults*: 5°C	5 days**	*Pupae*: Cost to adult female fecundity *Adults*: No cost in post-storage survival, female fecundity, or male mating capacity	Enriquez et al. (2020) [6]
Diptera	*Drosophila melanogaster*	Adults	6°C (21 hours) 1-hour ramp 22°C (1 hour) 1-h ramp	6°C 22°C	100 days**	*Males:* Benefit to fertility over 80 days *Females:* Increasing cost to fertility as duration of FTR exposure increases	Melicher et al. (2021) [32]
Hymenoptera	*Aphidius colemani*	Mummies	4°C (22 hours) 20°C (2 hours)	4°C	15 days	No cost to reproductive potential	Colinet et al. (2009) [48]
Hymenoptera	*Aphidius ervi*	Mummies	0, 4, or 7°C (22 hours) 20°C (2 hours)	0, 4, or 7°C	1 or 2 weeks	Benefit to female fecundity	Ismail et al. (2014) [33]
Hymenoptera	*Aphidius ervi*	Mummies	0 or 4 °C (22 hours) 20°C (2 hours)	0 or 4°C	4 weeks	Cost to lipid reserves, no cost to fitness at 0°C FTR	Ismail et al. (2013) [49]
Hymenoptera	*Aphidius ervi*	Mummies	7°C (22 hours) 20°C (2 hours)	7°C	Two weeks	No cost to fecundity, sex ratio, or mating capacity	Ismail et al. (2010) [50]

* FTR-exposed individuals relative to CLT-exposed individuals.
** For measurements associated with costs of FTR exposure.

life stages, there is either an inherent cost to the warm temperature exposure itself or there is a trade-off with the beneficial mechanisms activated during FTR.

New directions for research on fluctuating thermal regimes

Hypothesis 1. FTR repairs neuronal damage caused by chilling

As disruptions to ion and osmotic homeostasis persist, elevated levels of K^+ in the hemolymph promote extracellular depolarization and ultimately lead to cell death and a range of sublethal effects including neuromuscular dysfunction [1]. Recent work investigating the mechanisms of chill injury in *Locusta migratoria* has demonstrated the role hypokalemia plays in promoting an increase in intracellular Ca^{2+} and inducing cell death [34] as well as tissue-specific variation in chill-injury-induced programmed cell death via caspase-3 activity [35]. Two competing, but not mutually exclusive, hypotheses suggest that FTR functions to protect against or repair damage caused by chill injury [8]. The improvements in neuromuscular coordination of FTR-exposed insects, relative to CLT, suggest that FTR could act to inhibit these cell death mechanisms and/or act to repair damage downstream of their activation. *Megachile rotundata* prepupae [26] and pupae [16] under FTR have increased abundance of transcripts encoding neural patterning proteins, but to our knowledge, no other evidence of this relationship has been reported. Therefore, with a better understanding of the mechanisms that drive cell death during chill injury, follow-on experiments to test if FTR mitigates the activity of cell death mechanisms are now feasible.

Hypothesis 2. The periodicity of FTR acts as a zeitgeber to synchronize insect clocks

Besides the well-established role of FTR in aiding the recovery of membrane integrity and ion homeostasis, we hypothesize that the warm temperature pulse could also be functioning as a zeitgeber synchronizing the insects' clocks. Disruption of circadian mechanisms could lead to the desynchronization of daily activities and physiological processes [36]. There are two forms of circadian clocks, central and peripheral. The peripheral clocks are under various levels of regulation by the central clock and can have different physiological characteristics from those of the central clock [37]. Under certain environmental conditions, the peripheral clocks may become misaligned from the central clock [38]. Misalignment of the clocks can lead to deleterious impacts on an organism's physiology [39,40].

Insects' clocks function correctly only within a limited range of temperatures and photoperiods, outside these conditions, the insect's physiology can be negatively impacted. Exposure to sub- and super-optimal temperatures can significantly alter insects' rhythmicity [41–43]. The molecular underpinning for these alterations in rhythmicity appears to be changes in proteins levels for multiple clock genes (reviewed by Maguire and Sehgal [43]). The lack of a zeitgeber synchronizing the various clocks should be viewed as a stress due to its ability to alter insects' rhythmicity. Rearing *M. rotundata* under darkness and at constant 29°C results in the adults emerging randomly throughout the day and night, whereas exposing developing *M. rotundata* to a thermoperiod with an amplitude as little as 2°C synchronizes emergence to the beginning of the thermophase (i.e. the start of the temperature increase) and decreases the total number of days required for all adults to emerge [44]. Rearing adult *Drosophila simulans* under thermal profiles with a predictable (P) or nonpredictable (NP) temperature peak and a constant temperature (CT) control yielded a complex set of results [45]. The P line of flies had a significantly longer developmental time than either the CT or NP lines. The NP line had a significantly longer chill coma recovery time as compared with the CT and P lines. Finally, the CT flies had significantly larger body size than either of the two other lines of flies. It is clear from this study that untangling the possible role of the FTR warm pulse as a zeitgeber from its other physiological impacts will not be easy.

There are several important questions that need to be answered: 1) how stable is the temperature lower limit for maintaining rhythmicity — does it vary year to year or over development? 2) Are there developmental stages that are more sensitive than others to the effects of clock misalignment and if so, why? 3) What role does the duration of misalignment have on the overall outcome of the misalignment event? 4) Does having an ecologically relevant zeitgeber alter the thermal limit for maintaining rhythmicity? 5) What are the genes regulating the lower temperature limits for maintaining rhythmicity and what are the environmental cues that regulate them? Periodic arousals occur in hibernating mammals. These periodic increases in metabolic rate are thought to be governed by a clock mechanism that is independent of circadian control [46,47], and is instead regulated by metabolic rate, possibly the depletion of a key metabolic component [47]. While different in many aspects, mammalian hibernation and the insect FTR response may have interesting intersections in metabolic repair and endogenous cellular cycling.

Conclusions

Recent studies have driven forward our understanding of the physiological and molecular mechanisms that promote the neuromuscular dysfunction and cell death associated with chill injury [34,35]. A significant body of

literature now shows that the disruptions in ion homeostasis that promote these chill-injury phenotypes are mitigated by FTR exposure. Future inquiries into FTR's ability to reduce the intracellular influx of Ca^{2+} and cell death are now warranted. Recent measurements of the energetic and reproductive costs of FTR exposure have yielded complex results suggesting that negative consequences of FTR are life-stage-dependent. Finally, we propose additional work investigating the role FTR plays in repairing the damage caused by chill injury and coordinating circadian rhythm.

Data Availability

No data were used for the research described in the article.

Declaration of Competing Interest

None to declare.

Acknowledgements

This work was supported by the National Science Foundation Established Program to Stimulate Competitive Research NSF-EPSCoR-1826834 and the U.S. Department of Agriculture, Agricultural Research Service through project 3060-21220-03200D. The findings and conclusions in this publication are those of the authors and should not be construed to represent any official USDA or U.S. Government determination or policy. Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture. USDA is an equal-opportunity provider and employer.

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