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Biochemistry of trehalose accumulation in the spring field cricket, Gryllus veletis

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Abstract

The freeze tolerant spring field cricket, *Gryllus veletis*, accumulates trehalose in the blood and tissues during cold acclimation. Trehalose is the main blood sugar in insects, thus its blood concentration is tightly regulated, and trehalose is readily metabolized. How do crickets modify their metabolism to accumulate trehalose in their hemolymph and tissues? I hypothesized that trehalose production, transport, and consumption were modified during the cold acclimation to facilitate trehalose accumulation. Trehalose and the trehalose-specific transporter, TRET-1, are distributed among all tissues, and trehalose accumulates in the hemolymph, fat body, Malpighian tubules, and gut. Trehalose production increases during cold acclimation via increased glycogen phosphorylase activity, facilitating trehalose accumulation. However, trehalose transport and consumption are not modified during acclimation, indicating that they are not critical for trehalose accumulation. My study furthers the understanding of trehalose distribution and the mechanism of trehalose accumulation in a freeze tolerant insect.

Keywords

Summary for Lay Audience

Insect physiology is influenced by changes in temperature. Some insects mitigate the risks associated with low winter temperatures by changing their physiology to survive internal ice formation (i.e., are freeze tolerant). Many freeze tolerant insects accumulate molecules that protect cells and tissues from the direct effects of freezing. One cryoprotectant is the sugar trehalose, which is also the main insect blood sugar in insects. The spring field cricket, *Gryllus veletis*, accumulates trehalose in its blood and tissues when temperature and daylength decrease. Trehalose concentrations are usually tightly regulated, and it is readily metabolized into glucose for energy. I found that trehalose accumulates in all tissues except the brain and muscle during cold acclimation. How do crickets modify their metabolism to accumulate trehalose? To answer this question, I measured enzyme activity and gene expression associated with the production, transport, and consumption of trehalose. I found increased activity of glycogen phosphorylase, a key enzyme involved in trehalose synthesis, indicating that more trehalose is produced during acclimation. I found no change in expression of the trehalose transporter *Tret-1* with acclimation, indicating that an increase in transportation is not critical for trehalose accumulation. Finally, I saw no change in the activity of trehalase, the enzyme responsible for cleaving trehalose into two easily metabolized glucose monomers. I conclude that increased trehalose production likely drives trehalose accumulation in *G. veletis* during cold acclimation, even if trehalose breakdown and transport remain unchanged.

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Chapter 1

1   Introduction

As ectotherms, insects are greatly influenced by changes in temperature, which can have drastic effects on their physiology (Sinclair *et al.*, 2015). To mitigate the risks associated with low temperatures in autumn and winter, insects change their physiology to either avoid ice formation (freeze avoidant) or survive ice formation (freeze tolerant). In nature, changing seasons induce freeze tolerance in some insects due to gradually decreasing temperature and photoperiod (Sinclair *et al.*, 2015).

Freeze tolerant (FT) insects change their physiology during autumn to prevent or repair damage caused by low temperatures and freezing (Toxopeus and Sinclair, 2018). FT insects are hypothesized to control ice formation and structure by accumulating ice-nucleating agents and ice-binding proteins, to reduce the mechanical damage ice crystals may cause to cells and tissues (Toxopeus and Sinclair, 2018). Insects may also prevent and repair damage to macromolecules by accumulating cryoprotectants, intrinsically disordered proteins, and heat shock proteins (Toxopeus and Sinclair, 2018). Finally, FT insects may modify enzyme activity and amount to alter metabolic processes. During freezing, insects may decrease metabolic rate to reduce metabolic waste products, and increase production of antioxidants to reduce reactive oxygen species (Toxopeus and Sinclair, 2018).

Cryoprotectants are hypothesized to protect against the direct effects of freezing and include low molecular weight metabolites, lipids, ice-binding proteins, and transport proteins (Toxopeus and Sinclair, 2018). Cryoprotectants offer a range of different functions, including protecting macromolecules and controlling ice formation, and freeze tolerant (FT) insects may accumulate a combination of cryoprotectants (Toxopeus and Sinclair, 2018). Low molecular weight metabolites include sugar alcohols (polyols), sugars, and amino acids; these metabolites are hypothesized to stabilize macromolecules via hydrogen bonds, colligatively reduce ice content, and prevent protein aggregation by physically separating the molecules (Toxopeus and Sinclair, 2018). One example of a

sugar cryoprotectant is the disaccharide trehalose. Trehalose protects a wide variety of macromolecules in adverse conditions in a range of animals (Crowe *et al.*, 1992, 2001; Crowe, 2002), including freeze tolerant insects (Toxopeus *et al.*, 2019*b*).

1.1 Trehalose biochemistry and metabolism

Trehalose is a disaccharide composed of two glucose monomers connected by an α-1,1-glycosidic bond. This bond shapes the molecule like a clam shell, allowing trehalose to fit in close proximity to lipids and other macromolecules (Albertorio *et al.*, 2007). While in close proximity, trehalose can form hydrogen bonds and stabilize these molecules, due to its many hydroxyl groups (Crowe, 2002). Trehalose is the main blood sugar in insects, circulating in the hemolymph to provide energy to other tissues. Trehalose is non-reducing and resistant to acid hydrolysis, which facilitates its role as an energy carrier in the hemolymph as it will not readily interact with other molecules while circulating (Thompson, 2003).

1.1.1 Trehalose production and how it is regulated

Trehalose is mobilized from glycogen in the insect fat body (**Figure 1**). Glycogen mobilization is regulated by the corpora cardiaca in the brain, which produces hormones to either stimulate or inhibit glycogen synthesis (Lewis *et al.*, 1997). Hormones are produced by the brain and bind to receptors located on target tissues resulting in a signaling cascade (Lewis *et al.*, 1997). When hemolymph trehalose concentrations are low and energy is required, hypertrehalosemic hormone and adipokinetic hormone increase the activity of the rate-limiting enzyme glycogen phosphorylase increasing trehalose production (Steele, 1963). When hemolymph trehalose concentrations are high, insulin-like peptides increase the activity of glycogen synthase, reducing the amount of trehalose being synthesized (Roach *et al.*, 2012, **Figure 2**).

Glycogen is cleaved into glucose-1-phosphate monomers and converted into trehalose through the trehalose-6-phosphate synthase/phosphatase (TPSP) pathway. Glycogen phosphorylase (GP) is the first (and rate-limiting) enzyme in trehalose synthesis, converting glycogen into glucose-1-phosphate monomers (**Figure 1**). GP activity is regulated via phosphorylation, which produces rapid changes in activity relative to

protein synthesis and degradation. For example, bees increase GP activity by 20-80 % in only 15 seconds to power take off and flight (Storey, 2004). There are two forms of GP: the phosphorylated active form, GP*a*, and the dephosphorylated (less active) form, GP*b*. The ratio of GP*b*:GP*a* is maintained by Phosphorylase kinase (PK) and Phosphoprotein phosphatase-1 (PP1), which are also regulated by phosphorylation (Steele, 1982). In normal conditions, dephosphorylation of GP*a* and phosphorylation of GP*b* are in equilibrium to maintain GP activity, but PP1 is deactivated after 1-2 hours below c. 8 °C, increasing GP*a* activity (Storey, 2004; **Figure 3**). This increase in GP*a* increases overall GP activity and thus accelerates glycogen mobilization.
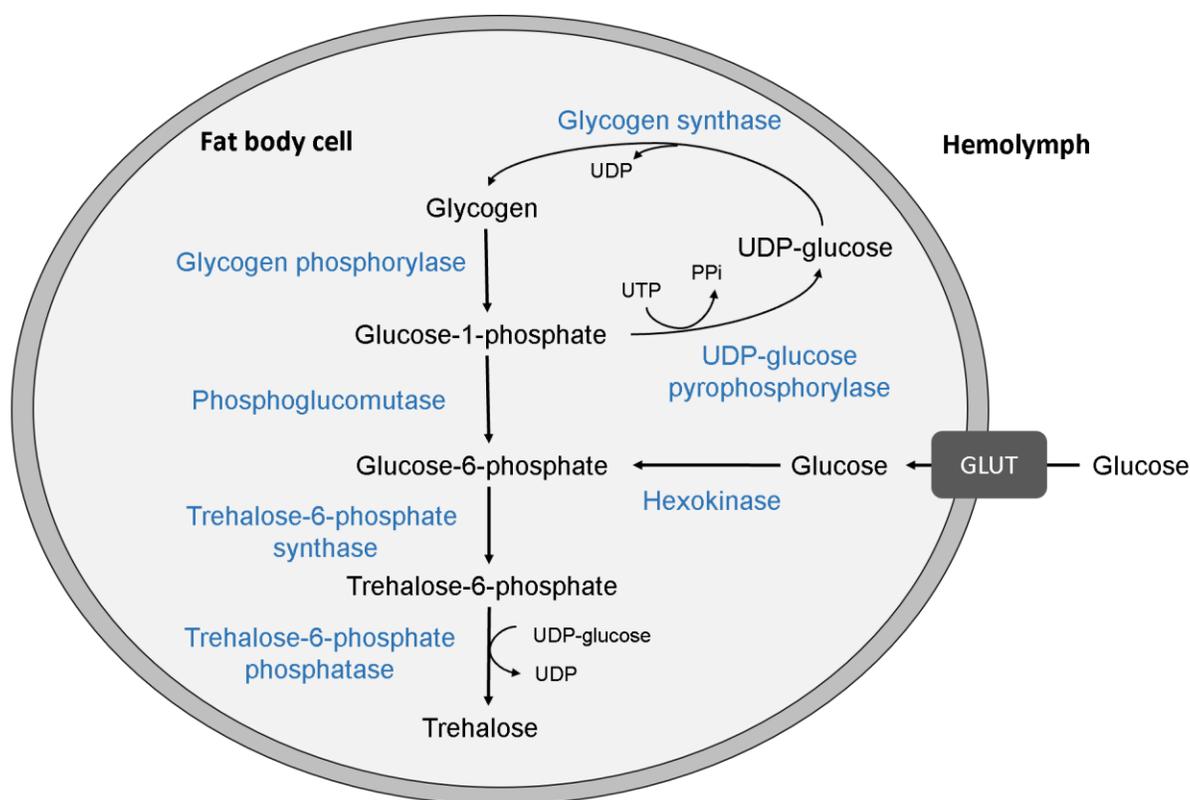
Figure 1. Trehalose synthesis pathway in insects. Glycogen can be converted through a series of reactions to form trehalose. Extracellular glucose can be imported and converted into glucose-6-phosphate. UDP, uridine diphosphate; UDP-glucose, uridine diphosphate-glucose; UTP, uridine triphosphate; PPi, pyrophosphate; GLUT, glucose transporter.
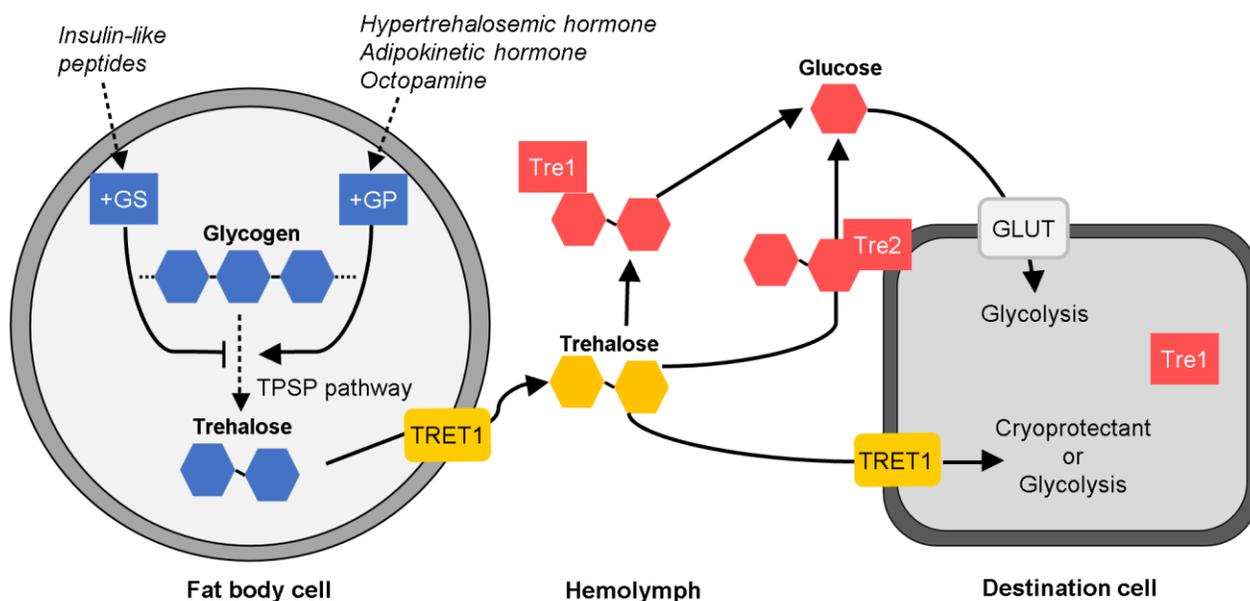
Figure 2. Overview of trehalose metabolism in insects. Trehalose synthesis is indicated in blue, trehalose transport is indicated in yellow, and trehalose consumption is indicated in red. Hormones produced in the brain bind to receptors on the fat body and trigger signaling cascades that can regulate glycogen phosphorylase and glycogen synthase activity. GP, glycogen phosphorylase; GS, glycogen synthase; TPSP, trehalose-6-phosphate synthase/phosphatase; TRET-1, trehalose-specific transporter; Tre1, trehalase-1; Tre2, trehalase-2; GLUT, glucose transporter
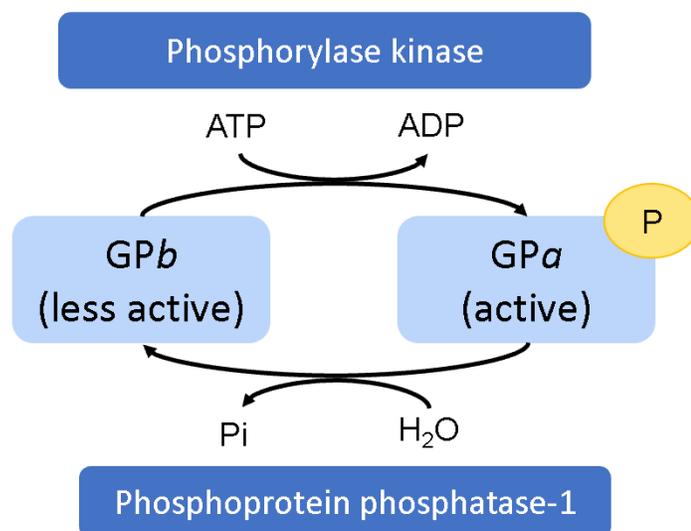
Figure 3. Mechanism of glycogen phosphorylase (GP) phosphorylation. Activity of GP is regulated through phosphorylation**.** Phosphorylase kinase phosphorylates the less active form of GP (GP*b*), producing the active form (GP*a)* and ADP. Phosphoprotein phosphatase-1 dephosphorylates GP*a* to produce GP*b*. ADP, adenosine diphosphate; ATP, adenosine triphosphate; Pi, inorganic phosphate.

1.1.2 Trehalose leaves and enters cells via the TRET-1 transporter

Once trehalose has been synthesized, it is exported from the fat body using the trehalose-specific transporter, TRET-1. Until TRET-1 was discovered (Kikawada *et al.*, 2007), it was believed that trehalose was broken down into glucose before entering the cell (Thompson, 2003). TRET-1 was first isolated and characterized as a trehalose-specific transporter in the sleeping chironomid, *Polypedilum vanderplanki* (Kikawada *et al.*, 2007). TRET-1 is a facilitated, bidirectional transporter that allows trehalose to move down its concentration gradient (Kikawada *et al.*, 2007). The transporter is a protein composed of 12 transmembrane helices, and functions independent of electrochemical membrane potentials and ATP availability (Kikawada *et al.*, 2007). When expressed in *Xenopus* oocytes, TRET-1 is specific to trehalose; it does not transport other disaccharides such as maltose or sucrose and does not transport anomers of trehalose such as neotrehalose and isotrehalose (Kikawada *et al.*, 2007). TRET-1 can transport the monomers composing trehalose (methyl-α-glucoside and 2-deoxyglucose), but does so at

c. 15 % the rate of trehalose (Kikawada *et al.*, 2007). TRET-1 is a high-capacity transporter, meaning the transporter is less likely to become saturated than other sugar transporters (Gupta and Gupta, 2020). TRET-1 from *P. vanderplanki* has a K_M (concentration of substrate required to reach ½ the maximum velocity) of 114.5 mM trehalose when expressed in *Xenopus* oocytes, which is orders of magnitude greater than the K_M of glucose transporters GLUT1 (3 mM glucose) and GLUT2 (17 mM glucose; Kikawada *et al.*, 2007).

TRET-1 orthologs have been identified in several orders including: Hymenoptera, Lepidoptera, Diptera, Coleoptera, Hemiptera, and Orthoptera (U.S. National Library of Medicine - NCBI, 2004). Although the amino acid sequences of TRET-1 are similar among insects, there is variation in the kinetic properties and tissue localization. The location of TRET-1 may be related to its function, and it may be present in areas where a lot of energy is required. In the Malpighian tubules of the brown plant hopper (*Nilaparvata lugens*), TRET-1 is a proton-dependent transporter which relates to its function of reabsorbing trehalose (Kikuta *et al.*, 2012). In general, TRET-1 is required in the fat body cells of all insects to export trehalose into the hemolymph and is required for import into other cells and tissues. In *P. vanderplanki,* TRET-1 is found in the fat body where trehalose accumulates during desiccation and protects macromolecules from the effects of dehydration. In *Bombyx mori*, *Tret-1* is expressed in the muscle, testis, and fat body, which may indicate that the main energy priority is to fuel flight (Kanamori *et al.*, 2010).

TRET-1 can be regulated through hormone signaling in response to environmental stresses and nutritional demands. When injected with exogenous insulin and AKH peptides, the kissing bug, *Rhodnius prolixus,* increased *Tret-1* abundance in the ovary (Leyria *et al.*, 2021). Furthermore, when the receptors for insulin and AKH were knocked down via RNA interference, TRET-1 abundance decreased indicating that both insulin and AKH are capable of regulating *Tret-1* expression (Leyria *et al.*, 2021). Some tissues may be tightly regulated through other hormones, such as nervous tissue which will take priority in terms of energy allocation during starvation stress. *D. melanogaster* increased *Tret-1* expression in the blood brain barrier (BBB) independent of insulin and AKH

action (Hertenstein *et al.*, 2021). However, knocking down Transforming growth factor β (TGF- β) via RNAi decreased *Tret-1* expression indicating that trehalose transport across the BBB of *Drosophila* is under TGF- β regulation (Hertenstein *et al.*, 2021).

Thus, TRET-1 is a trehalose-specific transporter in insects; however, there is diversity in the localization and kinetics of TRET-1 among insects and thus it is unclear if TRET-1 is distributed equally among all tissues, and if expression/kinetics are modified by adverse conditions.

1.1.3 Trehalose consumption

Once trehalose has been exported from the fat body to the hemolymph, it can be imported into other tissues that express TRET-1, or it can be cleaved into two glucose monomers by the trehalose-specific enzyme trehalase. Trehalase is a glycosidase which has been observed in bacteria, yeast, fungi, plants, insects, and some vertebrates (Shukla *et al.*, 2015). Insects have two forms of trehalase: Tre1 which is soluble, and Tre2 which is membrane-bound (Shukla *et al.*, 2015). The Tre1 enzyme has been isolated from the hemolymph, midgut, and egg of some insects, while Tre2 has been isolated in the flight muscles, ovary cells, spermatophore, midgut, brain, and thoracic ganglia (Shukla *et al.*, 2015).

Trehalase activity and mRNA abundance can be regulated by insulin and 20-hydroxyecdysone signaling pathways. In the pupae of Chinese Oak Silkworm (*Antheraea pernyi*), an increase in 20-hydroxyecdysone increased the activity of Tre1 and Tre2 and thus decreased hemolymph trehalose concentration (Li *et al.*, 2020). Injection of bovine insulin increased trehalase activity, whereas injection of a vector overexpressing the transcription factor forkhead box O (FoxO), which may be involved in insulin signaling as well as other signaling pathways, decreased trehalase activity (Li *et al.*, 2020).

The mechanism of regulating trehalose concentration can depend on environmental factors and physiological state. Insulin and 20- hydroxyecdysone (20-E) can alter hemolymph trehalose concentration in *Bombyx mori* larvae, but their effect/mechanism is altered by the feeding status of the larvae (Kh and Keshan, 2021). Both hormones

increased hemolymph trehalose concentration in fed larvae, and decreased hemolymph trehalose concentration in starved larvae, but the hormones differed in the mechanisms they used. In fed larvae, insulin increased GP activity and thus trehalose synthesis, whereas 20-E increased activity of both GP and trehalase. Although 20-E increased both trehalose synthesis and consumption, there was still an overall increase in hemolymph trehalose concentration, indicating that an increase in trehalose synthesis is sufficient to accumulate trehalose in *B. mori* larvae (Kh and Keshan, 2021). In starved larvae, insulin increased trehalase activity by increasing *Tre2* abundance and thus potentially increasing the amount of trehalase protein, while 20-E decreased GP activity and thus decreased trehalose synthesis. Although both hormones effected different parts of the trehalose metabolic pathway, they both resulted in a decrease in hemolymph trehalose concentration (Kh and Keshan, 2021).

Thus, trehalase can be modified because of adverse conditions and as a method to regulate trehalose concentration. However, it is unclear if trehalase can be modified by low temperatures, or if it is critical for regulating trehalose concentration.

1.2   Trehalose as a cryoprotectant

Alongside its role as the main blood sugar, trehalose is also hypothesized to play a role in cryoprotection and regulation of nutrient uptake in insects (Thompson, 2003). Much of our understanding of trehalose as a protectant molecule comes from studies of the anhydrobiotic nematode *Aphelenchus avenae*, tardigrades, and larvae of the sleeping chironomid, *Polypedilum vanderplanki* (Crowe *et al.*, 1992; Hengherr *et al.*, 2008; Sogame, 2017)*.* When desiccated slowly, *P. vanderplanki* larvae accumulate up to 20 % of their dry mass in trehalose (Watanabe *et al.*, 2003). There are two main hypotheses for how trehalose improves survival during anhydrobiosis: 1) trehalose acts as a water-replacement and forms hydrogen bonds with macromolecules, ensuring they maintain their structure and conformation, and 2) trehalose vitrifies, creating a glass-like substance, stabilizing macromolecules (Crowe *et al.*, 1992; Sakurai *et al.*, 2008). Trehalose is also hypothesized to improve survival of freeze avoidant and freeze tolerant insects. Trehalose may work alongside antifreeze proteins to protect cells and tissues. Antifreeze proteins prevent trehalose crystallization that may occur as trehalose

concentrations increase and temperatures decrease. As a result, trehalose remains soluble at low temperatures, ensuring that trehalose can still form hydrogen bonds with and protect macromolecules (Wen *et al.*, 2016).

Due to its chemical properties, trehalose interacts with a wide variety of macromolecules. Trehalose stabilizes phospholipid membranes by forming H-bonds with the phosphate head groups. This spatially separates the lipid chains, preventing the lipid membrane from undergoing a phase transition in anhydrobiotic conditions. In the absence of trehalose, a phase transition during desiccation can have several detrimental effects including separation of membrane components and membrane leakage (Crowe, 2002; Boothby, 2019). The mechanism of how trehalose protects proteins is less-understood, although it is hypothesized that trehalose replaces the H-bonds previously occupied by water molecules, preventing proteins from changing conformation or denaturing (Crowe, 2002). Non-reducing sugars, including trehalose, are capable of forming a glass-like state during dehydration where membranes and other macromolecules are immobilized, preventing them from coagulating and denaturing (Sakurai *et al.*, 2008). In *P. vanderplanki*, vitrification is required for larvae survival during desiccation as increasing the temperature to above the glass melting temperature decreases larvae survival (Sakurai *et al.*, 2008). Anhydrobiosis can also alter the activity of enzymes involved in trehalose metabolism. In *P. vanderplanki*, desiccation increases the activity of enzymes involved in trehalose synthesis including trehalose-6-phosphate synthase (TPS) and trehalose-6-phosphate phosphatase (TPP; homologous to TPSP in *G. veletis;* **Figure 2**; Mitsumasu *et al.*, 2010). Desiccation also decreased the activity of trehalase, decreasing trehalose consumption (Mitsumasu *et al.*, 2010).

Trehalose also accumulates in response to low temperatures and is not restricted to freeze tolerant insects. Several *Drosophila* species accumulate trehalose in response to low temperatures (Vesala *et al.*, 2012; Purac *et al.*, 2015). Trehalose also commonly accumulates in the hemolymph of freeze tolerant insects including the New Zealand alpine weta, *Hemideina maori.* The alpine weta increases hemolymph trehalose concentrations from c. 42 mM to c. 400 mM during cold acclimation (Neufeld and Leader, 1998). Low temperatures and desiccation can both result in osmotic stress, and in

both conditions, insects may accumulate trehalose to control water loss, thus maintaining cellular volume and osmotic gradients (Sinclair *et al.*, 2013). The main model for freezing in insects is that ice forms extracellularly; solutes are excluded from the ice, increasing extracellular osmotic pressure, causing cells to dehydrate via osmosis (Toxopeus and Sinclair, 2018). Freezing stops when the unfrozen liquid is in equilibrium with the frozen fraction, and thus ice can not form inside the cells due to their high osmotic pressure (Toxopeus and Sinclair, 2018). Accumulation of trehalose in the hemolymph and cells results in a high osmotic pressure, reducing the amount of ice required to form to achieve equilibrium (Toxopeus and Sinclair, 2018). Reducing ice content will also reduce water loss from cells, preventing cell shrinkage and molecular crowding (Toxopeus and Sinclair, 2018). Thus, trehalose can protect cells and tissues by directly interacting with them via hydrogen bonds, as well as indirectly through cellular dehydration.

1.3 *Gryllus veletis* as a model for freeze tolerance

The spring field cricket, *Gryllus veletis* (Orthoptera: Gryllidae), is widely distributed across North America (Alexander and Bigelow, 1960). *G. veletis* hatch in late summer and mature through a series of instars, and burrow under leaf litter to overwinter as a freeze-tolerant nymph. They then emerge in the spring and molt into adults to continue the life cycle (**Figure 4**; Alexander and Bigelow, 1960). In temperate regions, the changing of seasons causes gradual changes in temperature and photoperiod. We can simulate these environmental changes in the laboratory to induce freeze tolerance. *G. veletis* are moderately freeze tolerant; they can survive exposure to -12 °C for 90 min and can survive for seven days frozen at -8 °C (Toxopeus *et al.*, 2019*c*).
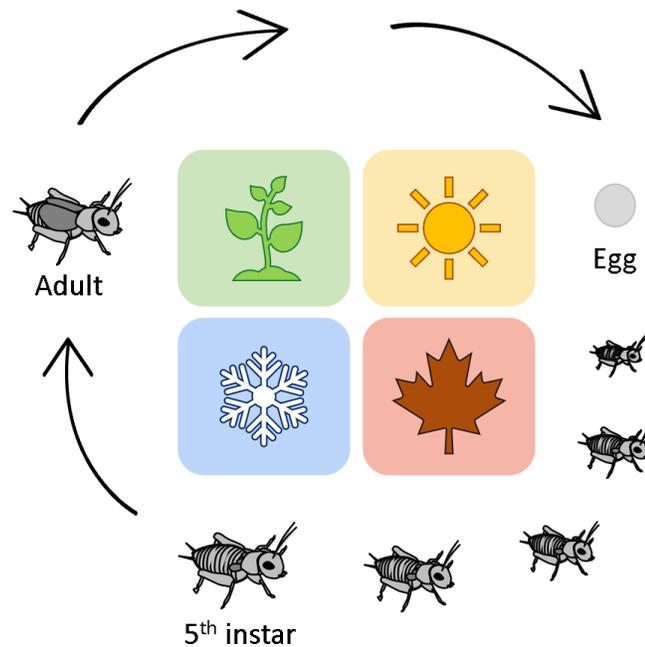
Figure 4. Life cycle of the spring field cricket, *Gryllus veletis*. Eggs hatch in late summer and grow through a series of instar stages. During the winter, 5th instar crickets burrow under leaf litter and overwinter as a freeze tolerant nymph. Crickets emerge in the spring and molt into adults. The green plant represents spring, the sun represents summer, the red leaf represents autumn, and the snowflake represents winter.

During laboratory acclimation, 5th instar nymphs are reared in gradually decreasing, fluctuating temperature and photoperiod over six weeks to induce freeze tolerance (Toxopeus *et al.*, 2019*c*). During the cold acclimation, the crickets decrease their metabolic rate by 33 %, and decrease water loss rate by 50 % (Toxopeus *et al.*, 2019*c*). During cold acclimation*, Gryllus veletis* accumulate trehalose, *myo*-inositol, and proline in the hemolymph and fat body. The concentrations of cryoprotectants in the hemolymph were 30 mM *myo*-inositol, 24 mM proline, and 70 mM trehalose. In the fat body, *myo*-inositol and proline increased in concentration by 10 nmol/mg tissue, while trehalose increased by 20 nmol/mg tissue relative to non-acclimated crickets (Toxopeus *et al.*, 2019*b*).

Accumulated trehalose appears to contribute to the freeze tolerance of *G. veletis*. *Gryllus veletis* injected with 1.3 M trehalose had increased survival when frozen at lower temperatures and for longer times relative to un-injected crickets (Toxopeus *et al.*, 2019*b*). Trehalose also increased cell survival when applied to fat body cells *ex vivo*, indicating it acts as a cryoprotectant. However, the presence of trehalose alone is not sufficient to induce freeze tolerance, other physiological changes associated with a cold acclimation are also required such as changes in gene expression and enzyme activity (Toxopeus *et al.*, 2019*b*).

The mechanism of trehalose cryoprotection in *G. veletis* likely is not solely concentration dependent, as different cryoprotectants accumulated are not functionally the same (Toxopeus *et al.*, 2019*b*). If the cryoprotectants have a colligative effect, an increase in any of the cryoprotectants would reduce ice content, improving animal and cell survival. However, *myo*-inositol, proline, and trehalose improved whole animal and tissue survival in different ways, indicating that each cryoprotectant may contribute to freeze tolerance in unique and over-lapping ways (Toxopeus *et al.*, 2019*b*). Trehalose may stabilize macromolecules by acting as a water replacement and preventing instability of proteins induced by low temperatures. Trehalose is a well-known protectant in anhydrobiosis, and so trehalose may increase survival and reduce damage to macromolecules during dehydration (Crowe *et al.*, 1992). Finally, trehalose is the main blood sugar in insects, and it may accumulate during acclimation to serve as an energy source to fuel recovery during thawing after freezing (Toxopeus *et al.*, 2019*b*).

During cold acclimation, *G. veletis* accumulate trehalose in the hemolymph and fat body, although the mechanisms underlying this accumulation are unclear. *Gryllus veletis* do not differentially express transcripts associated with trehalose synthesis or consumption, but there is an increase in abundance of the trehalose-specific transporter, TRET-1 in the fat body (**Table 1**; Toxopeus *et al.*, 2019*a*). However, it is unclear if any other tissues expressed *Tret-1*, and if *Tret1* abundance increases during the cold acclimation. Furthermore, it is unclear if trehalose metabolism is modified through enzyme activity, and if these changes facilitate trehalose accumulation during acclimation.

Table 1. **Transcripts related to trehalose metabolism in *Gryllus veletis* fat body and the status of gene expression during cold acclimation[a].**

Function	Description	mRNA abundance
Trehalose synthesis	Glycogen phosphorylase	No change
	Bifunctional T-6-P synthase/phosphatase	No change
	Glycogen synthase	No change
Trehalose transport	TRET-1	Increase
Trehalose consumption	Trehalase	No change
Hormone signalling	Insulin-like peptide receptor	Increase

[a]Transcriptome data adapted from (Toxopeus *et al.*, 2019*a*).

1.4 Objective and hypotheses

The first objective of my project is to determine the distribution of trehalose, TRET-1, and trehalase, as our knowledge of trehalose in *G. veletis* is limited to the fat body and hemolymph. The second objective of my project is to determine how *G. veletis* accumulates trehalose during cold acclimation. I will examine each stage of trehalose metabolism to determine how it is modified during acclimation.

I test three non-exclusive hypotheses about the mechanisms of trehalose accumulation:

1) Trehalose production increases during acclimation, facilitating trehalose accumulation. I predict there will be an increase in glycogen phosphorylase activity during cold acclimation.

2) Trehalose transport increases during acclimation, facilitating trehalose accumulation. I predict there will be an increase in *Tret-1* mRNA abundance in the fat body and tissues during cold acclimation.

3) Trehalose consumption decreases during acclimation, facilitating trehalose accumulation. I predict there will be a decrease in Tre1 and Tre2 activity during cold acclimation.

To determine if trehalose production is modified during acclimation, I measured the activity of glycogen phosphorylase in the fat body during acclimation as a measure for the rate of trehalose production. To determine if trehalose transport is modified during acclimation, I measured *Tret-1* mRNA abundance in the brain, fat body, Malpighian tubules, midgut and hindgut, and muscle in non-acclimated and 6-week acclimated crickets. Finally, to determine if trehalose consumption is modified during acclimation, I measured the activity of Tre1 and Tre2 in the hemolymph and tissues during acclimation (0-, 3-, and 6-week timepoints).

Chapter 2

2   Methods

2.1   Cricket rearing and acclimation

Our colony of spring field crickets originated from individuals collected in 2010 from the University of Lethbridge campus, Alberta, Canada, and were reared in incubators with modifications to the previously described protocol (Toxopeus *et al.*, 2019*c*). Crickets were housed in 50 L plastic containers ($59 \times 39 \times 30$ cm), and new cohorts were produced every two weeks. The crickets were fed rabbit food (Great Value, Walmart, London, ON, Canada), cat food (IAMS, Walmart, London, ON, Canada), fresh organic spinach, and water in tubes *ad libitum*. Paper fiber egg trays were provided in the bins as refuge, and small plastic trays containing sand and fine vermiculite were provided for egg laying. The crickets were reared in constant temperature and photoperiod (25 °C with 14L: 10D   photoperiod; Toxopeus *et al.*, 2019*c*).

To induce freeze tolerance, I exposed male, 5th instar crickets to fluctuating, gradually decreasing temperature and photoperiod. I placed 10-15 crickets from the main colony in a 6 L plastic container ($34 \times 21 \times 12$ cm) containing food and water *ad libitum*. I placed the plastic containers in Sanyo incubators (Sanyo MIR 154, Bensenville, Il, USA) and reared them in a 6-week cold acclimation as previously described (Toxopeus *et al.*, 2019*c*). Briefly, the crickets were reared in a fluctuating thermal regime over six weeks from 16/12 °C to 1/0 °C (daily high/low temperature). Photoperiod also decreased over the six weeks, from 11.5:12.5 to 7.9:16.1 L:D (Toxopeus *et al.*, 2019*c*). To confirm that the acclimated cohort was freeze tolerant (FT), I froze four individuals and held them at -8 °C for 1.5 h, allowed them to recover at 15 °C for 48 h, then measured the survival rate. I considered the cohort FT if 75 % of the frozen crickets survived (Toxopeus *et al.*, 2019*c*).

2.2   Tissue sampling

I dissected live 5th instar crickets and collected the hemolymph, brain, Malpighian tubules, fat body, midgut and hindgut, and thoracic muscle. I collected hemolymph by piercing the cricket under the pronotum with fine forceps and transferring the drop of hemolymph to a pre-weighed 1.5 mL microcentrifuge tube. I then pinned the crickets to a sylgard plate (184 silicone elastomer kit: Dow, MI, USA) using a pin through the thorax. I removed the hindlegs and head using a pair of scissors, to prevent the cricket from moving. The first tissue I collected was the brain; I transferred the head to a new sylgard plate and immobilized the head by placing a pin directly between the eyes. I then dissected and collected the brain using a pair of fine forceps and washed the tissue in a phosphate-buffered solution (PBS; Sigma Aldrich, Phosphate buffered saline, 10 mM phosphate buffer, 2.7 mM KCl, and 137 mM NaCl, pH 7.4 at 25 °C). The excess buffer was shaken off, and the tissue was placed in a pre-weighed microcentrifuge tube and placed on ice. I then dissected and collected the Malpighian tubules, fat body, midgut and hindgut, and thoracic muscle from the abdomen and thorax. The tissues were all washed with PBS and placed in microcentrifuge tubes as previously described. Once the dissections were completed, the tubes containing the tissues were weighed using a microbalance to determine the fresh mass (Mettler Toledo MX5, Zurich, Switzerland), before being frozen in liquid nitrogen vapour and stored at -80 °C until use in further assays. The exception to this were tissues collected for RNA extractions where I washed the tissues in PBS, placed them in pre-weighed microcentrifuge tubes, and added TRIzol (100 µL TRIzol per 5-10 mg tissue, ThermoFisher Scientific, Mississauga, ON, Canada) before freezing the solution in liquid nitrogen vapour and storing them at -80 °C until RNA extraction.

2.3   RNA extractions, DNase, and cDNA synthesis

To determine abundance of *Tret-1* mRNA, I extracted total RNA and synthesized cDNA from the brain, fat body, Malpighian tubules, midgut and hindgut, and thoracic muscle collected from non-acclimated and 6-week acclimated crickets. I thawed the collected tissues in TRIzol on ice and homogenized them using motorized pestles. I then extracted the RNA from the homogenate according to the manufacturer's instructions and

performed a DNase treatment to digest any remaining DNA (QuantaBio, Perfecta DNase 1 (RNase-free), Cat No. 95048-025), and reverse transcribed the purified RNA to generate cDNA (QuantaBio, qScript cDNA SuperMix, Cat. No.: 95048-025). Synthesized cDNA was stored at -20 °C until quantitative reverse-transcription PCR (qPCR) could be performed.

2.4 Quantitative real-time PCR (qPCR)

To determine mRNA abundance and distribution of *TRET-1,* I performed qPCR on the synthesized cDNA samples for brain, fat body, Malpighian tubules, midgut and hindgut, and thoracic muscle at 0- and 6-week acclimation timepoints. I generated primers specific to *Tret-1*, and the two reference genes *Elongation factor 1β (Ef1β)* and *Ribosomal protein L18 (Rpl18)* using the *G. veletis* transcriptome as reference (**Table 2**; Toxopeus *et al.*, 2019*a*). I diluted all cDNA samples to the same concentration prior to performing qPCR. To amplify the cDNA, I used SSO Advanced SYBR Green Supermix (Bio-Rad, Mississauga, ON, Canada) according to the manufacturer's instructions. Each qPCR included 10 µM forward and 10 µM reverse primer, and 200 ng cDNA, with each sample being run in triplicate using a Rotor-Gene Q real-time PCR cycler (Qiagen, Hilden, Germany). I normalized the cycle threshold (C_t) of each sample to a calibrator used in each run, then analyzed the results using the comparative C_t method. Briefly, I normalized the calibrated C_t average of each sample to the two reference genes (*Ef1β* and *Rpl18*), then calculated the relative transcript abundance to compare the non-acclimated and acclimated crickets. I compared the relative *Tret-1* mRNA abundance among tissues acclimation treatments using a two-way ANOVA and Tukey's post-hoc test.

Table 2. Primers used for quantitative PCR (qPCR) and cDNA synthesis, including the annealing temperature (T$_A$) used in qPCR cycle or cDNA synthesis, and the efficiency of the qPCR primers. Primers were designed using Primer3Plus; primers were designed to amplify a 75 – 200 base pair coding region of the mRNA, have a melting temperature of 60 °C, and a GC content of c. 50 – 60 %.

Target	Forward primer (5' to 3')	Reverse primer (5' to 3')	T$_A$ (°C)	Efficiency (%)
Elongation factor 1β	GGCTCAGCTGCTGTAGTGAA	CAGTGTCATCCTCCTCGTCG	55	95
Ribosomal protein l18	CGAGAAGCAAAACAAGGGGC	CACTTTTGCATGCACTCGCT	55	99
Trehalose transporter 1	TTGCCATAATCGCCGAGGTT	GGCAGTCCACTGAAGACGAA	55	101

2.5   Trehalose content and distribution

To determine trehalose content and distribution in *G. veletis,* I measured trehalose concentrations in the hemolymph, brain, fat body, Malpighian tubules, midgut and hindgut, and thoracic muscle. To determine if cold acclimation impacts trehalose content, I measured trehalose concentrations at 0-, 3-, and 6-week timepoints.

Briefly, I extracted tissues from the three acclimation timepoints the day of the experiment and determined trehalose concentration within one hour after dissection. I placed the extracted tissues in pre-weighed microcentrifuge tubes kept on ice and homogenized the tissues in 1:100 homogenization buffer (25 mM HEPES, 2 mM EDTA, and 0.1% Triton X-100, pH 7.8) using a motorized pestle. Homogenized samples were heated to 70 °C for 10 min to denature and inactivate any proteins present, then centrifuged at $20,000 \times g$ for 5 min to remove cellular debris. I transferred the supernatant to a new microcentrifuge tube and measured the trehalose concentration.

I measured trehalose concentration using the Megazyme trehalose assay kit (Neogen, Item No. K-TREH, Lansing, MI, USA). Briefly, I mixed 20 µL of supernatant with 232 µL of master mix containing hexokinase and glucose-6-phosphate dehydrogenase and incubated the mixture for 5 min at 30 °C while mixing. I read the absorbance of the plate as an endpoint assay, reading the absorbance at 340 nm at 30 °C to obtain a reference absorbance (abs$_0$). This absorbance measurement was taken to account for any glucose

already present in the sample. I then added 1 U of trehalase to each well and mixed the plate at 30 °C for 5 minutes. I measured the absorbance as an endpoint assay, reading the absorbance at 340 nm at 30 °C to obtain the final absorbance (abs_1).

To calculate trehalose concentration, I used Equation 1 to correct the absorbance of each sample:

$$Abs = abs_1 - abs_0$$

(Eqn. 1)

I then determined the concentration of trehalose in the sample using the standard curve as reference. I divided the trehalose concentration by the mass of the tissue collected during dissection. I was also able to calculate glucose concentrations in the samples using the reference absorbance, as it measured any glucose present in the sample. To calculate glucose concentration, I normalized the reference absorbance to my negative control, then used the trehalose standard curve to determine the initial trehalose concentration. Since every one trehalose molecule produces two glucose molecules, I converted the trehalose concentration to glucose by multiplying the concentration by two.

To compare trehalose and glucose concentrations among the acclimation treatments, I performed a one-way ANOVA and Tukey's post hoc test using GraphPad Prism (Ver. 9.3.1 for Windows, GraphPad Software, San Diego, California USA).

2.6 Glycogen phosphorylase activity

To determine GP activity during the cold acclimation, I collected fat body samples at 0-, 1.5-, 3-, and 6-week timepoints. Since glycogen is stored in the fat body, only fat body samples were collected and analyzed. I collected tissues the day of the assay, and measured GP activity within one hour of dissection with modifications to the previous protocol (Díaz-Lobo *et al.*, 2015). Briefly, I homogenized tissues in 1:20 ice cold homogenization buffer (25 mM HEPES, 2 mM EDTA, and 0.1% Triton X-100, pH 7.8) using a motorized pestle, and centrifuged the samples at $2,000 \times g$ for 15 min. I pipetted 98 µL of master mix containing 1 U of phosphoglucomutase (PGM) (Sigma Aldrich; Cat.

No. P3397, St. Louis, Missouri, USA) and 1 U of glucose-6-phosphate dehydrogenase (G-6-PDH) (Sigma Aldrich; Cat. No. G6378, St. Louis, Missouri, USA) onto a 96 well plate, and incubated it at 30 °C for 10 min.

I then added 2 µL of the homogenized sample to the wells and measured the change in absorbance as a kinetic assay at 30 °C and 340 nm for 1 hour, mixing the plate between each read. I plated each sample with three different master mixes: a background mix without glycogen to account for basal activity (V_{base}; 200 mM HEPES, 2.2 mM $NADP^+$, 1 mM $MgCl_2$, 5 uM glucose-1,6-bisphosphate, 10 mM NaH_2PO_4, pH 7.0), a reaction buffer including 4 mg/mL glycogen to measure overall GP activity ($V_{reaction}$), and a cAMP master mix containing 2 mM cAMP to activate GPb and measure maximum GP activity (V_{max}).

To calculate GPa activity, I used Equation 2:

$$GPa \left(\frac{U}{mgprotein} \right) = \frac{(Vreaction - Vbase) \times 100 \text{ µL} \times 20}{1000 \times \dfrac{6.22 l}{\dfrac{mmol}{cm}} \times 0.28 cm \times 2 \text{ µL} \times mg\ protein}$$

(Eqn. 2)

Where 100 was the final reaction volume, 20 was the dilution factor, 1000 was the conversion factor from mAU to AU, 6.22 l/mmol/cm was the extinction coefficient of NADPH at 340 nm, 0.28 was the path length, 2 was the volume of sample added.

To calculate the maximum GP activity, I used the same formula with the change of V_{max} – V_{base} for absorbance. To calculate the proportion of GPa relative to the max GP activity, I used Equation 3:

$$\% GPa = \frac{GPa\ activity\ (\frac{U}{mgprotein})}{Max\ GP\ activity\ (\frac{U}{mgprotein})} \times 100$$

(Eqn. 3)

To determine if GP activity was modified by the cold acclimation, I compared GP*a* activity among acclimation treatments using a two-way ANCOVA with mg protein as the co-variate and Tukey's post-hoc test. I also used a one-way ANOVA and Tukey's post hoc test to identify any changes in the proportion of GP*a* among acclimation treatments.

2.7 Glycogen content

To determine glycogen content in the fat body at 0-, 3-, and 6-week acclimation timepoints, I measured glycogen concentrations using homogenized samples collected for trehalase activity. The homogenized samples were stored at – 80 °C until glycogen could be measured. I thawed the samples on ice and used the supernatant to measure glycogen concentration using a protocol modified from my Glycogen Phosphorylase Activity assay (Section 2.6). Briefly, I mixed 100 µL of master mix (200 mM HEPES, 2.2 mM NADP$^+$, 1 mM MgCl$_2$, 5 uM glucose-1,6-bisphosphate, 10 mM NaH$_2$PO$_4$, 4 mg/mL glycogen, pH 7.0) containing 1 U PGM and 1 U G-6-PDH with 10 µL of sample supernatant on a 96 well plate. I mixed the plate for 5 min at 30 °C, then measured the absorbance as an endpoint assay at 340 nm at 30 °C to account for any glucose-1-phosphate initially present. I then added 10 µL of GP (Sigma Aldrich, Cat. No. P1261, St. Louis, Missouri, USA) to each well to initiate glycogen breakdown. I measured the change in absorbance as a kinetic assay for 15 min at 340nm at 30 °C.

To calculate glycogen concentration, I subtracted the initial absorbance from the final absorbance taken during the kinetic read and used a standard curve to convert the values into mg/mL. I then accounted for the reaction volume and divided the values by the mass of the tissue to determine glycogen amounts in mg glycogen/mg tissue. To determine if there were any changes among the acclimation treatments, I used a one-way ANOVA and Tukey's post hoc test.

2.8 Trehalase activity

To determine the combined activity of trehalase-1 and trehalase-2 during acclimation, I collected the hemolymph, brain, fat body, Malpighian tubules, midgut and hindgut, and thoracic muscle at 0-, 3-, and 6-week timepoints. I collected tissues the day of the assay,

and measured trehalase activity within one and a half hour of dissection using a protocol based off previous methods (Wegener *et al.*, 2010). Briefly, I homogenized tissues in 1:50 (1:100 for the brain) Ringer's solution (5 mM HEPES, 160 mM NaCl, 11 mM KCl, 8.4 mM CaCl$_2$, 5.9 mM MgCl, 4 mM glucose, pH 7.6) using a motorized pestle, and kept the samples on ice. I centrifuged the samples at $2,000 \times g$ for 10 min and transferred the supernatant to a new 1.5 mL microcentrifuge tube. The supernatant contained soluble (Tre1) and membrane bound (Tre2) trehalases. I then resuspended the pellet using ice cold homogenization buffer containing a detergent and a motorized pestle, to solubilize any remaining trehalases (25 mM HEPES, 2 mM EDTA, and 0.1% Triton X-100, pH 7.8).

I ran each sample with two buffers: 1) a background buffer of glucose assay reagent (V$_{base}$; 1.5 mM NAD, 1.0 mM ATP, 1 U hexokinase, 1 U G-6-PDH, with sodium benzoate and potassium sorbate as preservatives), 2) and a reaction buffer of glucose assay reagent and 20 mM trehalose (V$_{reaction}$). I mixed 250 uL of buffer with 10 uL of sample and measured the change in absorbance as a kinetic assay at 340 nm at 30 °C for 15 min. Trehalase activity was calculated using Equation 4:

$$\text{Trehalase activity (U/mg protein)} = \frac{(Vreaction - Vbase) \times 260 \ \mu L \times 50}{1000 \times \frac{6.22 l}{\frac{mmol}{cm}} \times 0.69 cm \times 10 \ \mu L \times mg \ tissue}$$

(Eqn. 4)

In this reaction, 260 µL represented the final reaction volume, 50 was the dilution factor (100 for brain), 1000 was to convert mAU to AU, 0.69 cm was the path length, 6.22 L/mmol/cm was the extinction coefficient for NADH at 340 nm, and 10 µL was the sample volume.

I compared the activity of Tre-1 and Tre-2 among acclimation treatments and tissues using a two-way ANOVA and Tukey's post hoc test.

Chapter 3

3 Results

3.1 Trehalose is present in all tissues, and accumulates during acclimation

I detected trehalose in the hemolymph, brain, fat body, Malpighian tubules, midgut and hindgut, and thoracic muscle (**Figures 5a and 5c**). Hemolymph trehalose concentration increased more than 300 % (**Figure 5a**), while glucose concentrations halved at 3- and 6-week cold acclimation timepoints (**Figure 5b**).

The brain and muscle trehalose concentration did not change throughout acclimation, but there was an increase in trehalose concentration in the fat body, Malpighian tubules, and gut (**Figure 5c**). Malpighian tubules and the gut increased trehalose concentration by 100% and 200 %, respectively, at both 3 and 6-week timepoints but fat body trehalose concentration only increased (by 150 %) at the 6-week timepoint (**Figure 5c**). Glucose concentrations decreased in all tissues by 50 – 100 % at the 3-week timepoint and remained low at the 6-week timepoint (**Figure 5d**).
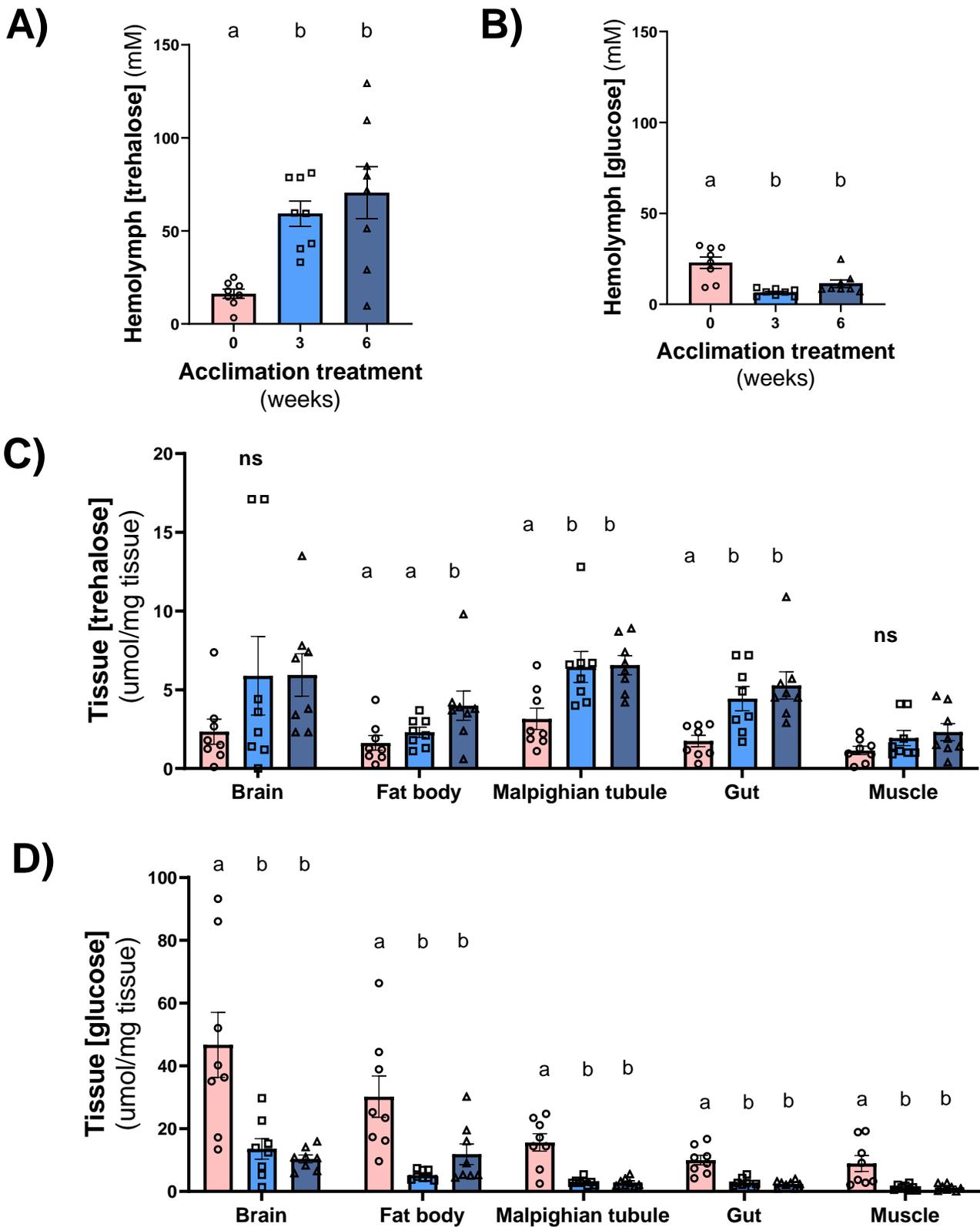
Figure 5. Trehalose and glucose are distributed among all *Gryllus veletis* tissues.
Mean ±S.E.M concentrations of trehalose (A and C) and glucose (B and D) measured in
5th instar *Gryllus veletis* in non-acclimated (pink bars), 3-week acclimated (light blue),
and 6-week acclimated (dark blue) crickets (n=8 crickets per timepoint). Different letters
denote significant differences via two-way ANOVA among acclimation treatments: A)
$F_{2,21} = 9.94$, $p < 0.001$, B) $F_{2,21} = 13.73$, $p < 0.001$, C) Brain $F_{2,21} = 1.47$, $p = 0.25$, fat
body $F_{2,21} = 3.77$, $p = 0.04$, Malpighian tubules $F_{2,21} = 6.26$, $p = 0.007$, gut $F_{2,21} = 7.01$, p
$= 0.005$, muscle $F_{2,21} = 1.80$, $p = 0.19$, D) Brain $F_{2,21} = 14.03$, $p < 0.001$, fat body $F_{2,21} =$
9.16, $p = 0.001$, Malpighian tubules $F_{2,21} = 20.10$, $p < 0.001$, gut $F_{2,21} = 19.86$, $p < 0.001$,
and muscle $F_{2,21} = 8.68$, $p = 0.002$.

3.2   Trehalose movement among *G. veletis* tissues

To determine if the increase in trehalose concentration was facilitated by an increase in
trehalose synthesis, and thus glycogen breakdown, I measured glycogen concentrations in
the fat body at 0-, 3-, and 6-week cold acclimation timepoints during acclimation. There
was no significant change in glycogen concentration during acclimation (**Figure 6**).
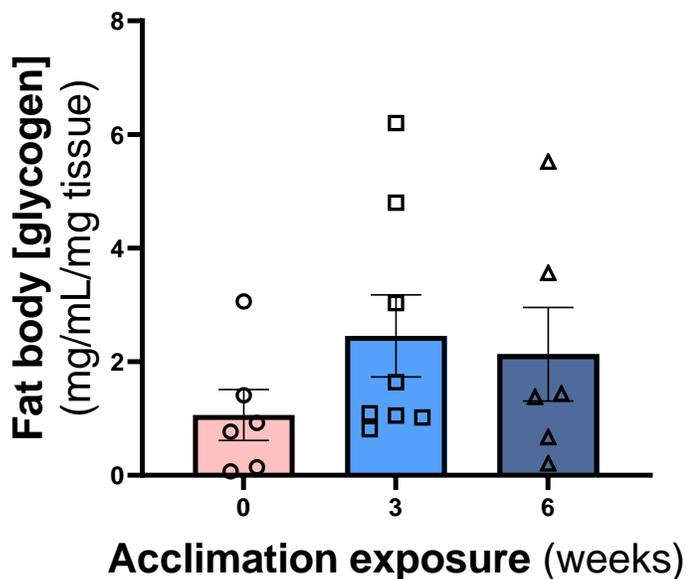
Figure 6. Glycogen concentrations in the fat body of 5ᵗʰ instar *Gryllus veletis* do not change during cold acclimation. Data presented are mean ± S.E.M, and there were no significant differences among acclimation treatments ($F_{2,17} = 1.07$, $p = 0.366$).

To examine the movement of trehalose from the site of production in the fat body to transport into cells and tissues, I examined the relationship between hemolymph and fat body trehalose concentration, and hemolymph and tissue trehalose concentration. Since trehalose moves down its concentration gradient through the transporter TRET-1, I predict that as fat body trehalose increases, trehalose is transported into the hemolymph resulting in an increase in hemolymph trehalose concentration. At 3- and 6-week timepoints during acclimation, there was a positive linear correlation between hemolymph and fat body trehalose concentration suggesting that hemolymph trehalose originated from the fat body (**Figure 7**).
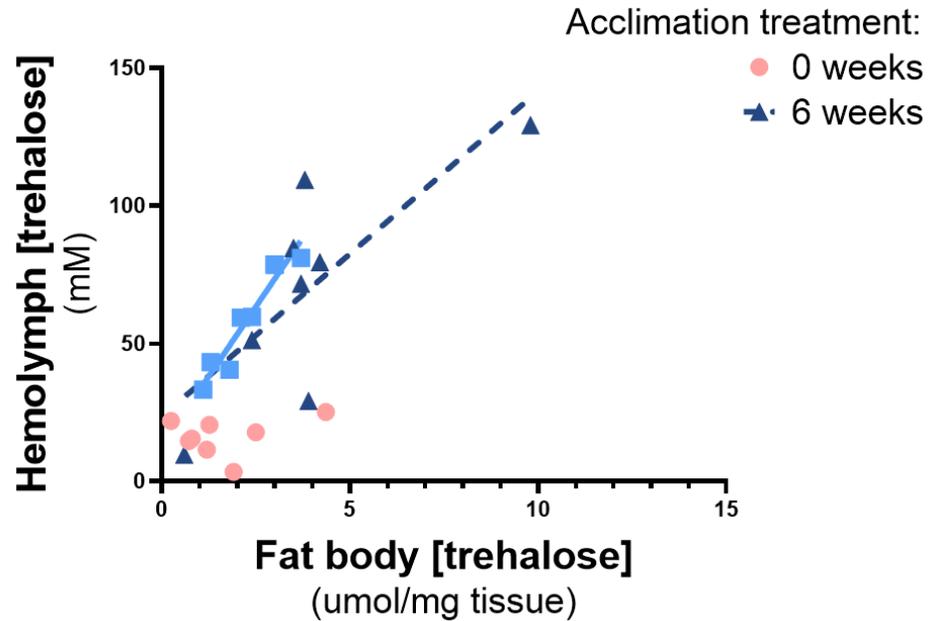
Figure 7. There was a positive correlation between fat body and hemolymph trehalose concentration in *G. veletis*. Individual data is presented, with lines of best fit representing a significant slope determined via simple linear regression. 0-week: $F_{1,6} = 0.47$, $p = 0.52$, $r^2 = 0.07$, 3-week: $F_{1,6} = 64.6$, $p < 0.001$, $r^2 = 0.91$, and 6-week: $F_{1,6} = 9.36$, $p = 0.02$, $r^2 = 0.61$.

To examine the gradient in hemolymph trehalose concentrations between the hemolymph and tissues, I examined the relationship between hemolymph trehalose concentration and the brain, Malpighian tubules, gut, and muscle (**Figure 6**). There was no significant correlation between the brain and hemolymph trehalose concentrations, supporting the results that trehalose does not accumulate in the brain (**Figure 6a**). There was a positive linear correlation between the Malpighian tubules and hemolymph, and the gut and the hemolymph at the 3-week timepoint (**Figures 8b and 8c**). There was a positive linear correlation at both the 3- and 6-week timepoints between the muscle and hemolymph trehalose concentrations (**Figure 8d**).
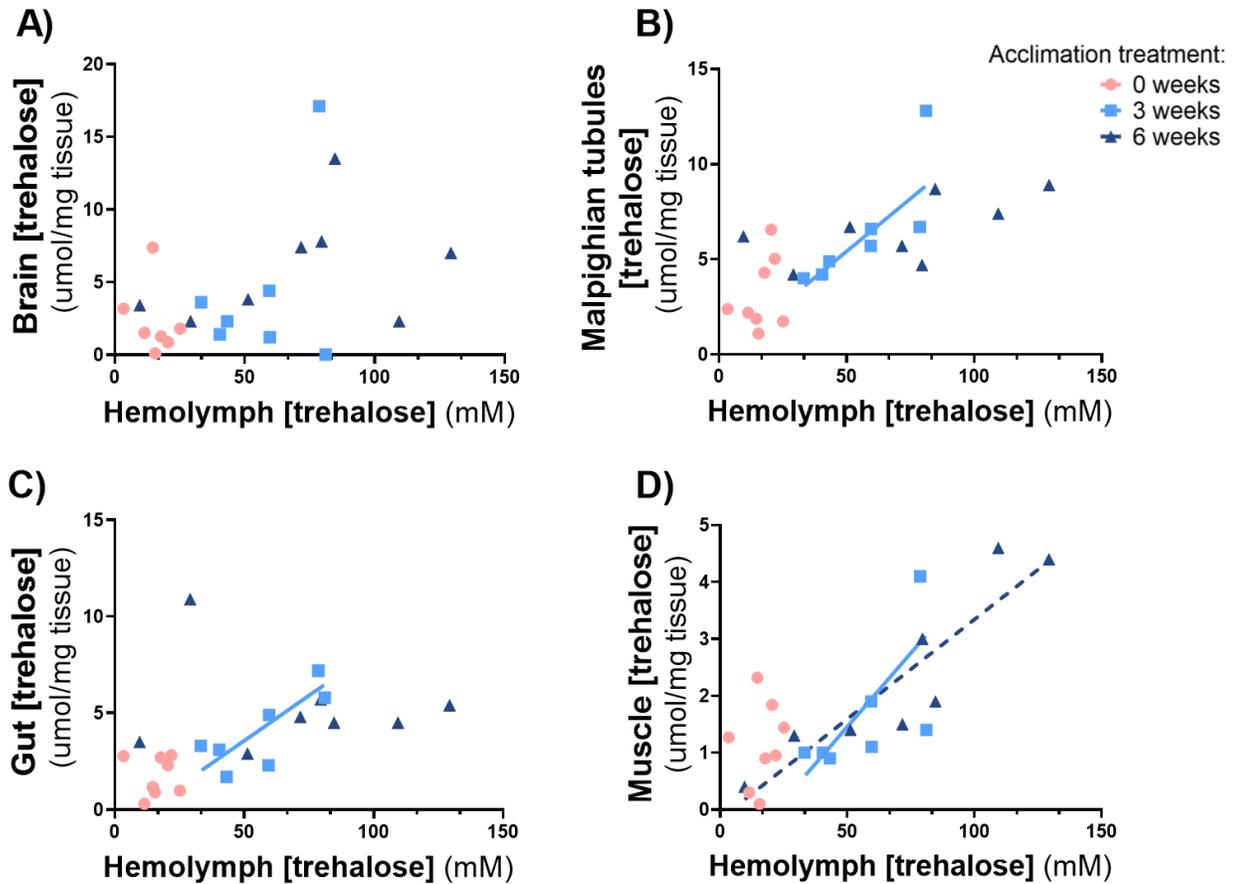
Figure 8. Relationship between hemolymph and tissue trehalose concentrations during cold acclimation in 5th instar *Gryllus veletis*. Individual data is plotted with lines of best fit denoting a significant slope determined via simple linear regression. A) Brain, no significant slopes, 0-week: $F_{1,5} = 0.36$, $p = 0.57$, 3-week: $F_{1,6} = 2.47$, $p = 0.17$, 6-week: $F_{1,6} = 0.96$, $p = 0.36$. B) Malpighian tubules, 3-week: $F_{1,6} = 7.9$, $p = 0.031$. C) Gut, 3-week: $F_{1,6} = 12.63$, $p = 0.012$. D) Muscle, 3-week: $F_{1,6} = 6.33$, $p = 0.045$, 6-week: $F_{1,6} = 28.54$, $p = 0.002$.

3.3 Glycogen phosphorylase activity increases during cold acclimation and is regulated by phosphorylation

To determine if trehalose production is altered during acclimation, I measured the activity of GP as a proxy for trehalose synthesis capacity. Glycogen phosphorylase *a* is the active form of GP, and so I measured GP*a* activity in the fat body at four timepoints during the cold acclimation to identify any changes in activity. GP*a* activity increased at the 3-week timepoint relative to the 1.5-week timepoint (**Figure 9a**). To determine the mechanism underlying this increase in activity, I measured the maximum GP activity to determine the proportion of GP*a* and GP*b* at each timepoint. There was an increase in the proportion of GP*a* at the 3-week timepoint (**Figure 9b**), indicating that the increase in GP*a* activity is due to an increase in the amount of GP*b* being phosphorylated and converted into GP*a*. There was no change in the maximum GP activity (**Figure 9c**), indicating that the increase in GP*a* activity is likely not due to an increase in the total amount of GP enzyme present, further supporting that GP activity is regulated through phosphorylation.
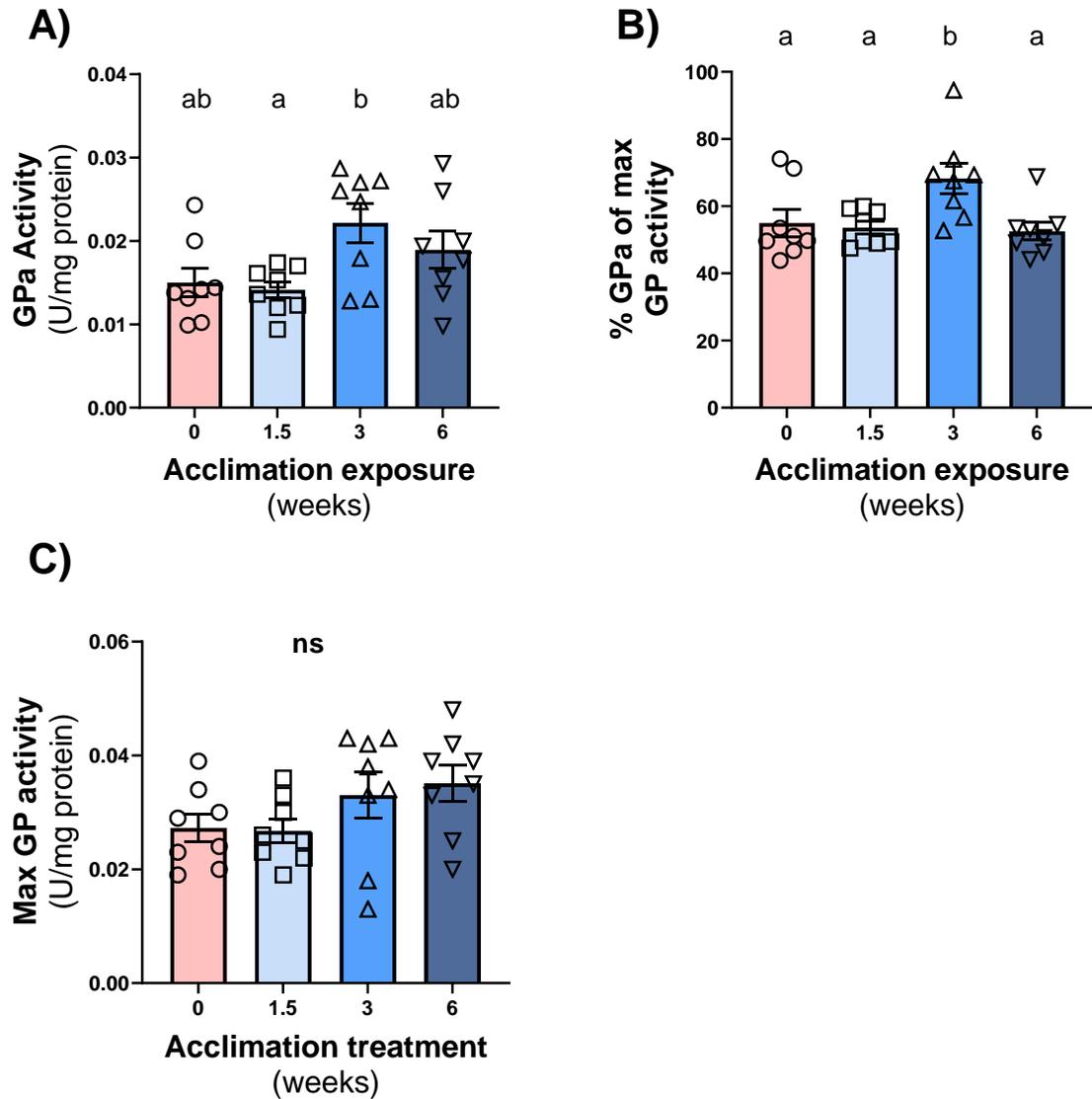
Figure 9. Glycogen phosphorylase activity increases in the fat body of *G. veletis* during a cold acclimation. Data presented are mean ± S.E.M. (n = 8 per timepoint). Different letters denote significant differences in glycogen phosphorylase (GP) activity, ns denotes no significant difference. A) There's an increase in GP activity at the 3-week timepoint relative to the 1.5-week timepoint: $F_{3,28} = 3.83$, $p = 0.02$. B) The proportion of GP*a* increases at 3-weeks relative to 1.5-weeks: $F_{3,28} = 4.58$, $p = 0.01$. C) There was no change in the maximum GP activity during acclimation: $F_{3,28} = 2.30$, $p = 0.098$.

3.4 Trehalose transporter is expressed in all tissues and increases in the brain during acclimation

To determine the distribution of *Tret-1* expression and if it changes during acclimation, I measured the mRNA abundance of *Tret-1* in non-acclimated and 6-week acclimated tissues. *Tret-1* was expressed in the brain, fat body, Malpighian tubules, gut, and muscle. I normalized *Tret-1* mRNA abundance relative to two references genes and determined that *Tret-1* mRNA abundance did not significantly differ among tissue and acclimation treatment (**Figure 10a**). I then determined the fold change in *Tret-1* mRNA abundance relative to the control group (0-week acclimated crickets) and found that *Tret-1* expression did significantly differ between cold acclimation treatments (**Figure 10b**).

3.5 Trehalase-1 and trehalase-2 activity

To determine if trehalase activity was modified during cold acclimation, I measured the activity of Tre1 and Tre2 during acclimation. I homogenized the tissue samples and measured the combined activity of Tre1 and Tre2 using the supernatant. Trehalase activity significantly differed among tissue and acclimation treatment, but there was no tissue × acclimation interaction indicating that acclimation had the same influence on trehalase activity in all tissues (**Figure 11a**). I re-suspended the pellet from the homogenized tissue using a detergent and measured the activity of the solubilized trehalases. Residual trehalase activity differed significantly among tissues, but not among acclimation treatments. Similarly, there was no tissue × acclimation interaction indicating that trehalase activity was not modified by the cold acclimation (**Figure 11b**). There was an increase in trehalase activity in the brain at the 6-week timepoint relative to the 3-week timepoint, indicating that the brain increased trehalose consumption during cold acclimation.
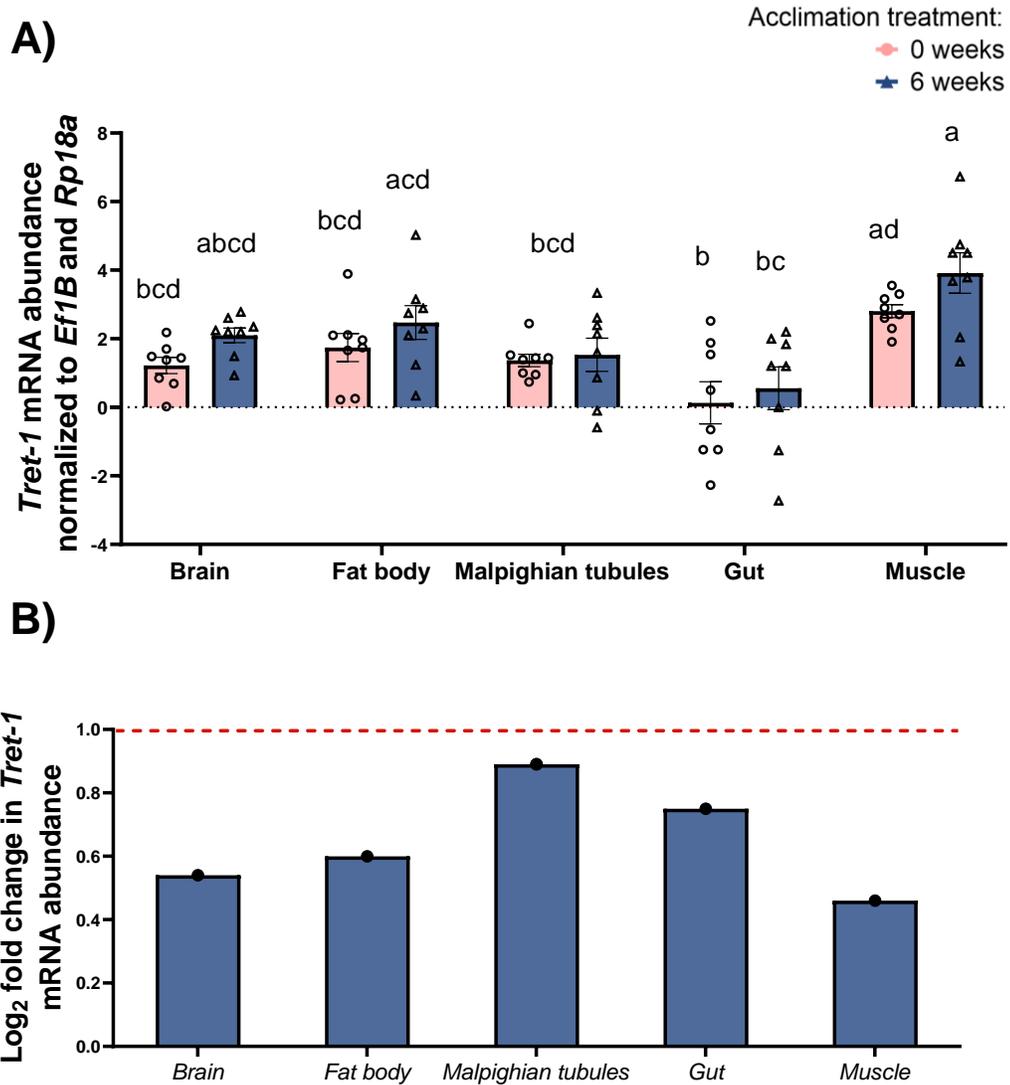
Figure 10. Distribution and abundance of *Tret-1* mRNA abundance in the tissues of acclimated and non-acclimated *Gryllus veletis*. A) Data presented are ΔCt values (*Tret-1* mRNA abundance relative to the two reference genes *Ef1β* and *Rpl18*) from non-acclimated (pink bars) and 6-week acclimated (blue) tissues. There was a significant effect of tissue ($F_{4,70} = 12.43$, $p < 0.001$) and acclimation treatment ($F_{1,70} = 5.68$, $p = 0.02$) on *Tret-1* abundance. B) Fold change in *Tret-1* expression in 6-week acclimated crickets relative to non-acclimated crickets. The dashed red line represents unchanged expression. There was a significant effect of cold acclimation on *Tret-1* abundance ($F_{1,4} = 20.95$, $p = 0.01$).
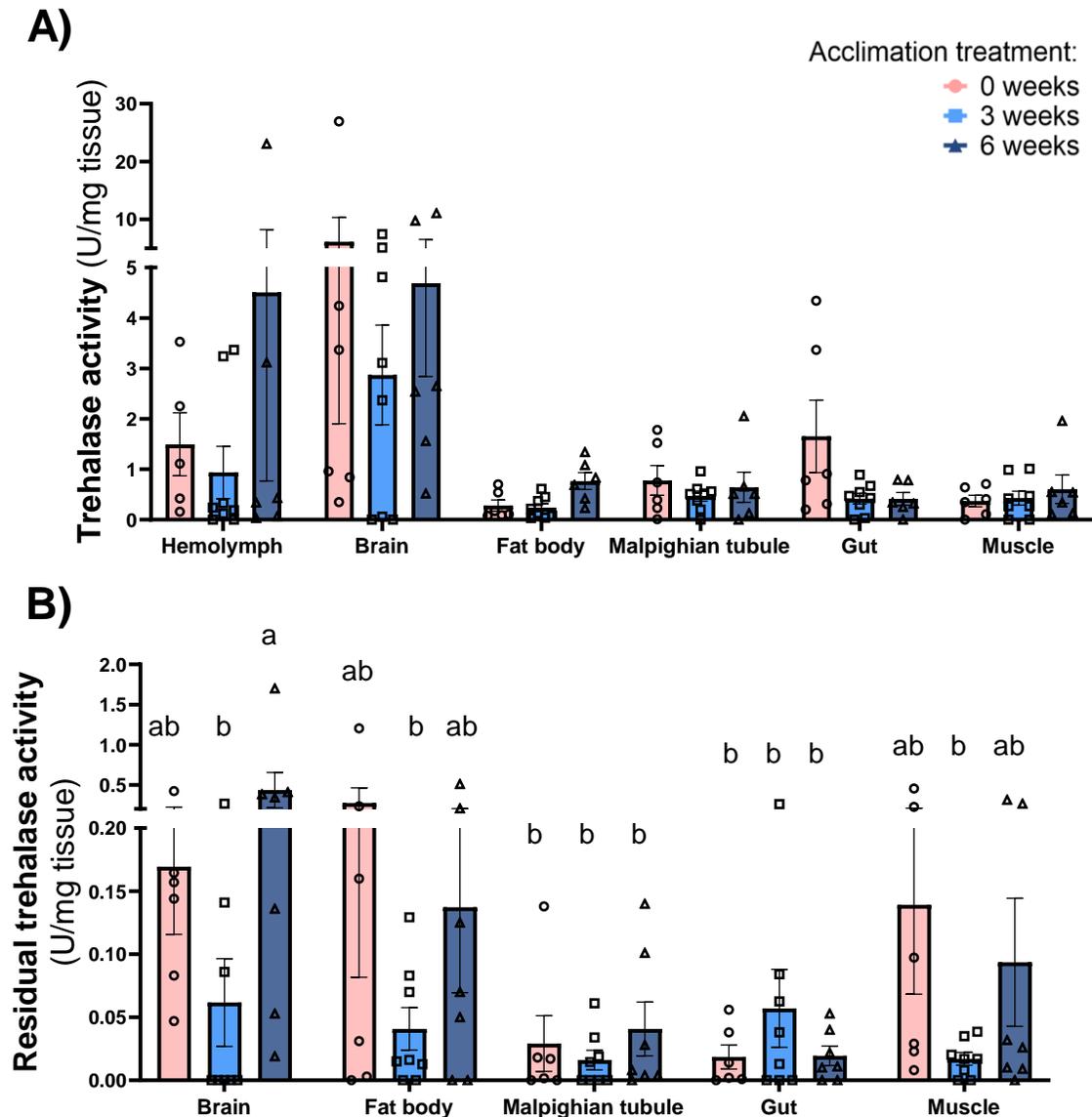
Figure 11. Activity of soluble (Tre1) and membrane bound (Tre2) trehalase in *Gryllus veletis* tissues during acclimation. Data presented are mean ± S.E.M., with letters denoting significant differences. A) Trehalase activity; trehalase activity significantly differed among tissues ($F_{5,101} = 4.58$, $p < 0.001$). B) Residual trehalase activity; trehalase activity significantly differed among tissues ($F_{4,90} = 3.33$, $p = 0.014$), and there was an increase in trehalase activity in the brain at 6-week timepoint ($p < 0.05$).

Chapter 4

4   Discussion

Freeze tolerant *Gryllus veletis* accumulate trehalose in the hemolymph and fat body during cold acclimation (Toxopeus *et al.*, 2019*b*), however it was unclear how this accumulation occurred because trehalose concentrations are normally tightly regulated to meet physiological and metabolic demands (Thompson, 2003). My aim was to identify the distribution of trehalose and *Tret-1* among tissues and determine how the trehalose metabolic pathway was modified during a cold acclimation. I hypothesized that trehalose production, transport, and consumption were modified during the cold acclimation to facilitate trehalose accumulation. I found that trehalose and TRET-1 were distributed among all tissues, and that trehalose accumulated in the hemolymph, fat body, Malpighian tubules, and gut during cold acclimation. Glycogen phosphorylase activity increased during acclimation (**Figure 9)**, suggesting that trehalose synthesis increased, facilitating trehalose accumulation. *Tret1* expression was not changed during acclimation, which suggests that an increase in trehalose transport is not required for trehalose concentrations to increase (**Figure 10)**. This indicates that an increase in *Tret-1* abundance may not be critical for trehalose accumulation during cold acclimation. Finally, trehalase activity did not change in all tissues during cold acclimation (**Figure 11)**. This suggests that a decrease in trehalase activity is not critical for trehalose to accumulate during acclimation. Furthermore, residual trehalase activity increased in the brain during acclimation, which suggests that the role of trehalose may be tissue-specific, where some tissues may prioritize metabolizing trehalose over accumulating it (**Figure 11b)**.

Trehalose is the main blood sugar in insects and as a non-reducing sugar is able to circulate in the hemolymph (Thompson, 2003). I predicted that trehalose would be present in all tissues due to its role as an energy source, and the findings of this study support this prediction. Prior to this study, trehalose accumulation had only been reported in the fat body and hemolymph in *Gryllus veletis* (Toxopeus *et al.*, 2019*b*). I determined that trehalose was also distributed in the brain, Malpighian tubules, gut, and muscle **(Figures 5a and 5c)** and accumulates in the Malpighian tubules and the gut (**Figure 5c**).

Since trehalose does not accumulate in all tissues, the role of trehalose may be tissue-specific; it may serve as a cryoprotectant or fuel reserve in some tissues but may be used for basal metabolism in others. Trehalose metabolism and accumulation in response to low temperatures has been studied in several insect orders; however, many studies only measure trehalose concentrations in the hemolymph (Kim *et al.*, 2017; León-Quinto and Serna, 2022) or whole insect homogenates (Fields *et al.*, 1998; Mitsumasu *et al.*, 2010; Izadi *et al.*, 2019). Results from this study suggest that the role of trehalose is tissue-specific, and thus measurements at the whole insect level, or in single tissues, may result in overgeneralizations of trehalose metabolism.

Trehalose is synthesized in the fat body in insects by cleaving glycogen via glycogen phosphorylase (GP). GP is the rate limiting enzyme in the trehalose pathway, and a known target for hormonal regulation to control trehalose concentration (Steele, 1982; Roach *et al.*, 2012). I measured GP*a* activity as a proxy for trehalose synthesis and found that GP*a* activity increases during acclimation (**Figure 9a**). This indicates there is an increase in glycogen breakdown and an increase in trehalose production, which supports my first hypothesis that increased trehalose production facilitates trehalose accumulation. This is similar to previous studies, where an increase in GP activity increased hemolymph trehalose concentration (Kh and Keshan, 2021). Interestingly, although there is no increase in GP activity during the first three weeks of acclimation there is still an increase in trehalose concentration in hemolymph and certain tissues (**Figures 5a and 5c**). A possible explanation for this is that GP activity remains constant during this time, but glycogen stores are being replenished by converting glucose into glycogen via the hexokinase and the trehalose-6-phosphate synthase/phosphatase (TPSP) pathway (Roach *et al.*, 2012). In *Leptinotarsa decemlineata*, trehalose-6-phosphate synthase is expressed in the fat body, foregut, hindgut, trachea, ovaries, and testes indicating that other tissues may be capable of synthesizing trehalose (Shi *et al.*, 2016). This is supported by my finding that glucose concentrations in the hemolymph and tissues decreased significantly in the first three weeks of acclimation (**Figures 5b and 5d**). This is similar to a previous study in diapausing wheat midge (*Sitodiplosis mosellana*), where trehalose concentrations increased and remained high during diapause (a state of dormancy that some insects enter during the winter), but glucose concentrations decreased and remained low (Huang *et al.*,

2021). Another explanation is that the crickets are still eating at the beginning of the acclimation, so they replenish glycogen stores from their environment. There is an increase in GP activity at the 3-week timepoint during acclimation, which indicates that although GP may not be responsible for the initial increase in trehalose concentration, it may be required to maintain these high levels of trehalose concentration. Further studies may wish to inhibit glycogen phosphorylase activity using a pharmacological inhibitor to determine if GP is critical for trehalose synthesis, or if other energy sources are able to compensate and maintain trehalose concentration. Furthermore, GP is a site of hormonal regulation by adipokinetic hormone and hypertrehalosemic hormones (Sun *et al.*, 2002; Huang *et al.*, 2012). Further studies may wish to knockdown the receptor for these hormones via RNA interference to further our understanding of how these hormones may be regulating GP activity in *G. veletis* during cold acclimation.

Once trehalose has been synthesized in the fat body, it is exported to the hemolymph through the trehalose-specific transporter, TRET-1 (Kikawada *et al.*, 2007). TRET-1 is a facilitated transporter which moves trehalose down its concentration gradient, which is supported by my study. When trehalose concentration in the fat body increased, trehalose moved down its concentration gradient into the hemolymph, increasing hemolymph trehalose concentration (**Figure 7**). Once in the hemolymph, trehalose can continue down its concentration gradient into cells and tissues if TRET-1 is present. In *G. veletis*, *Tret-1* was expressed in all tissues studied, and the movement of trehalose into these tissues was supported by the relationships between hemolymph and tissue trehalose concentration, as well as previous literature (**Figure 8**, Kikawada *et al.*, 2007).My second hypothesis was that trehalose transport was increased during acclimation, facilitating trehalose accumulation. While there was an effect of tissue and acclimation on *Tret-1* mRNA abundance, there was no interaction between tissue and acclimation which indicates that the tissues will express *Tret-1* regardless of acclimation treatment. One explanation for this may be that TRET-1 is a high-capacity transporter and is capable of transporting high concentrations of trehalose without becoming saturated, and thus more protein is not required to cope with increased trehalose concentration (Kikawada *et al.*, 2007). Furthermore, this accumulation occurs over the course of six weeks, and thus transport into tissues may occur slowly over time. Another possibility is that TRET-1 is not

transcriptionally regulated, and so further studies are required to localize and quantify TRET-1 protein during the acclimation. TRET-1 may be regulated through post-translational modifications, which may be investigated further by examining phosphorylation and acetylation sites. Further studies may also wish to knockdown expression of *Tret-1* via RNA interference to determine if TRET-1 is critical for trehalose accumulation.

I hypothesized that trehalose consumption in the hemolymph and tissues will decrease during acclimation, facilitating trehalose accumulation. During acclimation, trehalose accumulated in the hemolymph, fat body, Malpighian tubules, and gut (**Figure 5**); however, trehalase activity did not change in these tissues during acclimation, which does not support my third hypothesis (**Figure 11**). This indicates that the increase in trehalose production was sufficient for trehalose to accumulate in these tissues. These results are not supported by previous literature; *S. mosellana* sampled during fall and winter increased trehalose concentrations, but decreased Tre1 activity (Huang *et al.*, 2021). Similar results were found in *P. vanderplanki*, where trehalose concentrations increased and trehalase activity decreased during desiccation (Mitsumasu *et al.*, 2010). However, regulation of trehalose metabolism during a cold acclimation may vary by insect, as well as by tissue. In *G. veletis*, trehalase activity may be required to fuel metabolic activity during acclimation, and thus may not be critical to trehalose accumulation. In the brain, there was an increase in residual trehalase activity, which suggests that the brain is actively metabolizing trehalose during cold acclimation. The main source of energy in the brain are carbohydrates, in the form of trehalose in insects (Rittschof and Schirmeier, 2017). The brain is critical for maintaining function of and regulating other tissues, and thus would likely be metabolically active during cold acclimation. I detected trehalose, *Tret-1*, and trehalase in the brain, indicating that trehalose is transported across the blood brain barrier, and is metabolized for energy. In *B. mori* larvae, insulin-like peptides (ILPs) and 20-hydroxyecdysone (20-E) can regulate trehalase activity (Kh and Keshan, 2021); future studies can knock down ILP and 20-E receptors in *G. veletis* to determine their role in trehalase regulation.

The fat body is the site of trehalose synthesis, but it may also accumulate trehalose as a cryoprotectant, to protect itself from the effects of freezing. This is supported by previous literature, where trehalose increased the survival of *G. veletis* fat body *ex vivo* (Toxopeus *et al.*, 2019*b*). Additionally, the fat body may maintain a high trehalose concentration to ensure the concentration gradient persists and directs synthesized trehalose into the hemolymph. The Malpighian tubules and the gut are important sites for ion and water homeostasis, and may accumulate trehalose as a cryoprotectant to ensure they maintain function at low temperatures, or to serve as a fuel source during freezing and recovery (Storey and Storey, 1986; MacMillan *et al.*, 2012). The brain and muscle do not accumulate trehalose during the acclimation (**Figure 5c**); however, *Tret-1* is expressed in both tissues and thus they may be metabolizing trehalose during the acclimation and require energy to maintain basal physiological processes regardless of environmental conditions. To further the understanding of the role of trehalose during acclimation, future studies can isotopically label the cricket food and follow the metabolic pathway, to determine if the ingested carbohydrates are used to replenish glycogen stores or used directly in glycolysis (McConnell and Antoniewicz, 2016). Further studies should also extend the observation period to include a freezing and thawing event, to determine if trehalose is being used to fuel the recovery process (Boardman *et al.*, 2011; MacMillan *et al.*, 2012).

4.1 Conclusion: a model of trehalose accumulation in *G. veletis*

In my thesis, I address the distribution of trehalose and *Tret-1* and built a model for how trehalose metabolism is modified during a 6-week cold acclimation (Figure 12). Trehalose was detected in all tissues, and accumulates in the hemolymph, fat body, Malpighian tubules, and gut. There was an increase in trehalose production, facilitating its accumulation. Trehalose transport and consumption were not modified during the acclimation, and thus may not be critical for trehalose accumulation (**Figure 10**). Trehalose is produced in the fat body, and travels down its concentration gradient into the hemolymph, increasing hemolymph trehalose concentration. Hemolymph trehalose will

then continue down its concentration gradient into cells and tissues, increasing tissue trehalose concentration.

Thus, this study furthers our understanding of trehalose distribution and the mechanism of trehalose accumulation in a freeze tolerant insect. Findings from this study expand our knowledge of *Gryllus veletis* as a model for freeze tolerance and may be used to guide future studies in manipulating trehalose metabolism.
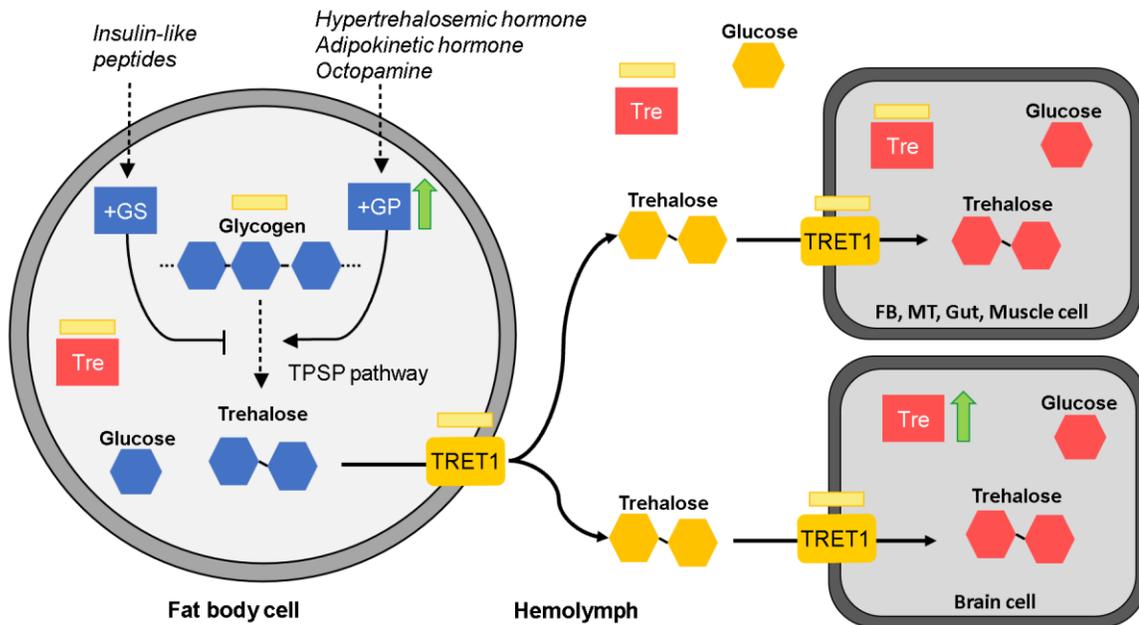
Figure 12. Summary of changes in trehalose metabolism during acclimation in *G. veletis*. Trehalose production, transport, and consumption are summarized, with green arrows indicating an increase in enzyme activity, and yellow bars representing no change in enzyme activity or mRNA abundance. GP, glycogen phosphorylase; GS, glycogen synthase; TPSP, trehalose-6-phosphate synthase/phosphatase; TRET-1, trehalose-specific transporter; FB, fat body; MT, Malpighian tubules; Tre, trehalase. In summary, there was an increase in GP activity in the fat body, no change in *Tret-1* abundance, and an increase in trehalase activity in the brain.

Bibliography

Albertorio F, Chapa VA, Chen X, Diaz AJ, Cremer PS. 2007. The α,α-(1→1) Linkage of Trehalose Is Key to Anhydrobiotic Preservation. Journal of the American Chemical Society **129**, 10567–10574.

Alexander RD, Bigelow RS. 1960. Allochronic speciation in field crickets, and a new species, Acheta veletis. Evolution **14**, 334–346.

Boardman L, Terblanche JS, Sinclair BJ. 2011. Transmembrane ion distribution during recovery from freezing in the woolly bear caterpillar Pyrrharctia isabella (Lepidoptera: Arctiidae). Journal of Insect Physiology **57**, 1154–1162.

Boothby TC. 2019. Mechanisms and evolution of resistance to environmental extremes in animals. EvoDevo **10**, 30.

Crowe LM. 2002. Lessons from nature: the role of sugars in anhydrobiosis. Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology **131**, 505–513.

Crowe JH, Crowe LM, Oliver AE, Tsvetkova N, Wolkers W, Tablin F. 2001. The trehalose myth revisited: Introduction to a symposium on stabilization of cells in the dry state. Cryobiology **43**, 89–105.

Crowe JH, Hoekstra FA, Crowe LM. 1992. Anhydrobiosis. Annual Review of Physiology **54**, 579–599.

Díaz-Lobo M, Garcia-Amorós J, Fita I, Velasco D, Guinovart JJ, Ferrer JC. 2015. Selective photoregulation of the activity of glycogen synthase and glycogen phosphorylase, two key enzymes in glycogen metabolism. Organic & Biomolecular Chemistry **13**, 7282–7288.

Fields PG, Fleurat-Lessard F, Lavenseau L, Febvay, Gérard, Peypelut L, Bonnot G. 1998. The effect of cold acclimation and deacclimation on cold tolerance, trehalose and free amino acid levels in Sitophilus granarius and Cryptolestes ferrugineus (Coleoptera). Journal of Insect Physiology **44**, 955–965.

Gupta N, Gupta V (Eds.). 2020. *Experimental Protocols in Biotechnology*. New York, NY: Springer US.

Hengherr S, Heyer AG, Köhler H-R, Schill RO. 2008. Trehalose and anhydrobiosis in tardigrades - evidence for divergence in responses to dehydration. FEBS Journal **275**, 281–288.

Hertenstein H, McMullen E, Weiler A, Volkenhoff A, Becker HM, Schirmeier S. 2021. Starvation-induced regulation of carbohydrate transport at the blood–brain barrier is TGF-β-signaling dependent. eLife.

Huang J-H, Bellés X, Lee H-J. 2012. Functional Characterization of Hypertrehalosemic Hormone Receptor in Relation to Hemolymph Trehalose and to Oxidative Stress in the Cockroach Blattella germanica. Frontiers in Endocrinology **2**, 1–11.

Huang Q, Zhang G, Nan J, Cheng W, Zhu-Salzman K. 2021. Characterization of trehalose metabolic genes and corresponding enzymatic activities during diapause of Sitodiplosis mosellana. Journal of Insect Physiology **135**, 104324.

Izadi H, Mohammadzadeh M, Mehrabian M. 2019. Cold Tolerance of the Tribolium castaneum (Coleoptera: Tenebrionidae), Under Different Thermal Regimes: Impact of Cold Acclimation. Journal of Economic Entomology **112**, 1983–1988.

Kanamori Y, Saito A, Hagiwara-Komoda Y, Tanaka D, Mitsumasu K, Kikuta S, Watanabe M, Cornette R, Kikawada T, Okuda T. 2010. The trehalose transporter 1 gene sequence is conserved in insects and encodes proteins with different kinetic properties involved in trehalose import into peripheral tissues. Insect Biochemistry and Molecular Biology **40**, 30–37.

Kh SD, Keshan B. 2021. The feeding status regulates the action of insulin and 20-hydroxyecdysone on haemolymph trehalose level in Bombyx larvae. Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology **255**, 110579.

Kikawada T, Saito A, Kanamori Y, Nakahara Y, Iwata K -i., Tanaka D, Watanabe M, Okuda T. 2007. Trehalose transporter 1, a facilitated and high-capacity trehalose transporter, allows exogenous trehalose uptake into cells. Proceedings of the National Academy of Sciences of the United States of America **104**, 11585–11590.

Kikuta S, Hagiwara-Komoda Y, Noda H, Kikawada T. 2012. A Novel Member of the Trehalose Transporter Family Functions as an H+-Dependent Trehalose Transporter in the Reabsorption of Trehalose in Malpighian Tubules. Frontiers in Physiology **3**, 1–9.

Kim Y, Lee D-W, Jung JK. 2017. Rapid Cold-Hardening of a Subtropical Species, Maruca vitrata (Lepidoptera: Crambidae), Accompanies Hypertrehalosemia by Upregulating Trehalose-6-Phosphate Synthase. Environmental Entomology **46**, 1432–1438.

León-Quinto T, Serna A. 2022. Cryoprotective Response as Part of the Adaptive Strategy of the Red Palm Weevil, Rhynchophorus ferrugineus, against Low Temperatures. Insects **13**, 134.

Lewis DK, Jezierski MK, Keeley LL, Bradfield JY. 1997. Hypertrehalosemic hormone in a cockroach: molecular cloning and expression. Molecular and Cellular Endocrinology, 101–108.

Leyria J, El-Mawed H, Orchard I, Lange AB. 2021. Regulation of a Trehalose-Specific Facilitated Transporter (TRET) by Insulin and Adipokinetic Hormone in Rhodnius prolixus, a Vector of Chagas Disease. Frontiers in Physiology **12**, 1–21.

Li Y-N, Liu Y-B, Xie X-Q, Zhang J-N, Li W-L. 2020. The Modulation of Trehalose Metabolism by 20-Hydroxyecdysone in Antheraea pernyi (Lepidoptera: Saturniidae) During its Diapause Termination and Post-Termination Period. Journal of Insect Science **20**, 18.

MacMillan HA, Williams CM, Staples JF, Sinclair BJ. 2012. Reestablishment of ion homeostasis during chill-coma recovery in the cricket *Gryllus pennsylvanicus*. Proceedings of the National Academy of Sciences of the United States of America **109**, 20750–20755.

McConnell BO, Antoniewicz MR. 2016. Measuring the Composition and Stable-Isotope Labeling of Algal Biomass Carbohydrates via Gas Chromatography/Mass Spectrometry. Analytical Chemistry **88**, 4624–4628.

Mitsumasu K, Kanamori Y, Fujita M, Iwata K, Tanaka D, Kikuta S, Watanabe M, Cornette R, Okuda T, Kikawada T. 2010. Enzymatic control of anhydrobiosis-related accumulation of trehalose in the sleeping chironomid, Polypedilum vanderplanki: Trehalose metabolism in the desiccating sleeping chironomid. FEBS Journal **277**, 4215–4228.

Neufeld DS, Leader JP. 1998. Freezing survival by isolated Malpighian tubules of the New Zealand alpine weta Hemideina maori. Journal of Experimental Biology **201**, 227–236.

Purac J, Kojić D, Petri E, Popovic Z, Grubor-Lajsic G, Blagojević D. 2015. Cold Adaptation Responses in Insects and Other Arthropods: An "Omics" Approach. Short views on Insect Genomics and Proteomics.89–112.

Rittschof C, Schirmeier S. 2017. Insect models of central nervous system energy metabolism and its links to behavior. Glia **66**.

Roach PJ, Depaoli-Roach AA, Hurley TD, Tagliabracci VS. 2012. Glycogen and its metabolism: some new developments and old themes. Biochemical Journal **441**, 763–787.

Sakurai M, Furuki T, Akao K, Tanaka D, Nakahara Y, Kikawada T, Watanabe M, Okuda T. 2008. Vitrification is essential for anhydrobiosis in an African chironomid, *Polypedilum vanderplanki*. Proceedings of the National Academy of Sciences of the United States of America **105**, 5093–5098.

Shi J-F, Xu Q-Y, Sun Q-K, Meng Q-W, Mu L-L, Guo W-C, Li G-Q. 2016. Physiological roles of trehalose in Leptinotarsa larvae revealed by RNA interference of trehalose-6-phosphate synthase and trehalase genes. Insect Biochemistry and Molecular Biology **77**, 52–68.

Shukla E, Thorat LJ, Nath BB, Gaikwad SM. 2015. Insect trehalase: Physiological significance and potential applications. Glycobiology **25**, 357–367.

Sinclair BJ, Coello Alvarado LE, Ferguson LV. 2015. An invitation to measure insect cold tolerance: Methods, approaches, and workflow. Journal of Thermal Biology **53**, 180–197.

Sinclair BJ, Ferguson LV, Salehipour-shirazi G, MacMillan HA. 2013. Cross-tolerance and Cross-talk in the Cold: Relating Low Temperatures to Desiccation and Immune Stress in Insects. Integrative and Comparative Biology **53**, 545–556.

Sogame Y. 2017. Current findings on the molecular mechanisms underlying anhydrobiosis in Polypedilum vanderplanki. Current Opinion in Insect Science, 6.

Steele JE. 1963. The site of action of insect hyperglycemic hormone. General and Comparative Endocrinology **3**, 46–52.

Steele JE. 1982. Glycogen phosphorylase in insects. Insect Biochemistry **12**, 131–147.

Storey KB (Ed.). 2004. *Functional metabolism: regulation and adaptation*. Hoboken, N.J: John Wiley & Sons.

Storey JM, Storey B. 1986. Winter survival of the gall fly, Eurosta solidaginis: Profiles of fuel reserves and cryoprotectants in a natural population. Journal of Insect Physiology **32**, 549–566.

Sun D, Garcha K, Steele JE. 2002. Stimulation of trehalose efflux from cockroach (Periplaneta americana) fat body by hypertrehalosemic hormone is dependent on protein kinase C and calmodulin. Archives of Insect Biochemistry and Physiology **50**, 41–51.

Thompson SN. 2003. Trehalose - The Insect 'Blood' Sugar. Advances in Insect Physiology **31**, 205–285.

Toxopeus J, Des Marteaux LE, Sinclair BJ. 2019*a*. How crickets become freeze tolerant: The transcriptomic underpinnings of acclimation in Gryllus veletis. Comparative Biochemistry and Physiology Part D: Genomics and Proteomics **29**, 55–66.

Toxopeus J, Koštál V, Sinclair BJ. 2019*b*. Evidence for non-colligative function of small cryoprotectants in a freeze-tolerant insect. Proceedings of the Royal Society B **286**, 20190050.

Toxopeus J, McKinnon AH, Štětina T, Turnbull KF, Sinclair BJ. 2019*c*. Laboratory acclimation to autumn-like conditions induces freeze tolerance in the spring field cricket Gryllus veletis (Orthoptera: Gryllidae). Journal of Insect Physiology **113**, 9–16.

Toxopeus J, Sinclair BJ. 2018. Mechanisms underlying insect freeze tolerance: Mechanisms of insect freeze tolerance. Biological Reviews **93**, 1891–1914.

Vesala L, Salminen TS, Koštál V, Zahradníčková H, Hoikkala A. 2012. Myo-inositol as a main metabolite in overwintering flies: seasonal metabolomic profiles and cold stress tolerance in a northern drosophilid fly. Journal of Experimental Biology **215**, 2891–2897.

Vickery, V.R., and Kevan, D.K.M. PS and PC. 1985. *The grasshoppers, crickets and related insects of Canada and adjacent regions : Ulonata: Dermaptera, Cheleutoptera, Notoptera, Dictuoptera, Grylloptera, and Orthoptera*. Agriculture Canada.

Watanabe M, Kikawada T, Okuda T. 2003. Increase of internal ion concentration triggers trehalose synthesis associated with cryptobiosis in larvae of Polypedilum vanderplanki. Journal of Experimental Biology **206**, 2281–2286.

Wegener G, Macho C, Schloder P, Kamp G, Ando O. 2010. Long-term effects of the trehalase inhibitor trehazolin on trehalase activity in locust flight muscle. Journal of Experimental Biology **213**, 3852–3857.

Weissman DB, Gray DA. 2019. Crickets of the genus Gryllus in the United States (Orthoptera: Gryllidae: Gryllinae). Zootaxa **4705**, 1–277.

Wen X, Wang S, Duman JG, *et al. 2016. Antifreeze proteins govern the precipitation of trehalose in a freezing-avoiding insect at low temperature. Proceedings of the National Academy of Sciences of the United States of America **113**, 6683–6688.

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