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Low-temperature injury in insect tissues and mechanisms of its repair

PhD. Thesis

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České Budějovice 2020

This thesis should be cited as:

Štětina, T. (2013) Low-temperature injury in insect tissues and mechanisms of its repair. Ph.D. Thesis Series, No. 14. University of South Bohemia, Faculty of Science, School of Doctoral Studies in Biological Sciences, České Budějovice, Czech Republic, 83 pp.

Annotation

While physiological adjustments accompanying insect cold acclimation prior to cold stress have been relatively well explored, processes of recovery after cold stress received much less attention. Using a broad array of physiological, molecular biological and microscopic techniques, I aimed to capture the complexity of processes occurring after the exposure to cold stress in the larvae of two drosophilid species – freeze tolerant *Chymomyza costata* and freeze sensitive *Drosophila melanogaster*. Several lines of evidence pointed toward impaired mitochondrial functions in lethally frozen larvae. The freeze-tolerant phenotypes of *C. costata* retain integrity of mitochondria even after deep freezing.

Declaration [in Czech]

Prohlašuji, že svoji disertační práci jsem vypracoval samostatně pouze s použitím pramenů a literatury uvedených v seznamu citované literatury.

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České Budějovice, 1. 6. 2020

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This thesis originated from a partnership of Faculty of Science, University of South Bohemia, and Institute of Entomology, Biology Centre of the ASCR, supporting doctoral studies in the Physiology and Developmental Biology study programme.

Přírodovědecká fakulta
Faculty of Science

Jihočeská univerzita v Českých Budějovicích
University of South Bohemia in České Budějovice

Financial Support

GAČR projects No.: 13-01057S, 16-06374S, 19-13381S

Student grants GAJU of the University of South Bohemia:
04-038/2014/P, 04-159/2016/P, 04-037/2019/P

RVO KZO 103-2020

Acknowledgements

My biggest thanks belongs to my supervisor, Vladimír Koštál, for his constant patience and his dedication during the assistance of writing the thesis, which helped me improve the quality of the thesis significantly. Further, I want express my gratitude to the current and former members of our lab, who took a great part in the projects presented in the thesis. A special thanks belongs to Jaroslava Korbelová for her never-ending support and patience and love during the difficult task, as well as to the members of my family and my friends.

List of papers and author's contributions

The thesis is based on following papers (listed chronologically):

I. Štětina T., Hůla P., Moos M., Šimek P., Šmilauer P., Košťál V (2018). Recovery from supercooling, freezing, and cryopreservation stress in larvae of the drosophilid fly, *Chymomyza costata*. Scientific Reports 8, 4414. (IF: 4.525)
TŠ participated in experimental design, performed analyses of potassium concentrations, CO_2 production, helped in microarray design and production, conducted microarray hybridizations, processed the datasets, and wrote parts of manuscript.

II. Štětina T., Poupardin R., Moos M., Šimek P., Šmilauer P., Koštál V. (2019). Larvae of *Drosophila melanogaster* exhibit transcriptional activation of immune response pathways and antimicrobial peptides during recovery from supercooling stress. Insect Biochemistry and Molecular Biology 105, 60-68. (IF: 3.618)
TŠ participated in experimental design, performed acclimations and samplings, conducted microarray and bioinformatic analyses, designed figures, helped in interpretation of results, and wrote parts of manuscript.

III. Štětina, T., Des Marteaux, L.E., Koštál, V. Insect mitochondria as targets of freeze-induced injury. (manuscript in preparation)
TŠ participated in experimental design, conducted most of the experiments, including TEM imaging and subsequent ImageJ analysis, CS activity assays, respiration analyses using PreSens and Oroboros systems, PCR analyses, designed the figures, helped in interpretation of results, and wrote the manuscript together with VK.

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Contents

1. Introduction

Climate of our planet is characterized by dramatic seasonality caused by tilt of the Earth axis of rotation at 23.5° angle relative to orbital plane. Periodical occurrence of winter in non-equatorial climate zones poses two main challenges on organisms: insufficiency or absence of food and low temperatures or even deep frosts. Small ectotherms such as insects need to profoundly change their phenotype in order to cope with winter conditions. Most insects are unable to migrate over long distances (continental scale). They are also relatively poor regulators of body temperature (unlike endotherms). Hence, most insects survive winter in a dormant state – diapause (analogy of endotherm hibernation). Diapause enables insects to prepare for and survive harsh winter conditions due to a complex physiological reconstruction of their bodies. Insects usually enter into diapause at the end of summer when ambient temperature is still relatively high. Diapausing insects first cease development (immatures) or reproduction (adults), they accumulate energy reserves, and switch their metabolism to 'economy' mode. The next set of physiological adjustments comes together with cold acclimation during gradual decline of ambient temperature. The most important outcome of all these phenotypic alterations is acquisition of high cold resistance. Many overwintering insects can survive even at extremely low body temperatures (deep below 0°C) for relatively long periods of time (weeks to months) – a capacity not seen in any endotherm hibernator. For instance, body temperature of some insects overwintering in interior Alaska may fall below -50°C (Carrasco et al., 2012).. Physiological limits of some overwintering insects go even below the lowest temperature ever recorded in Antarctica (-89.2 °C) (Turner et al., 2009; Koštál et al., 2011b). Such fantastic adaptation merits scientific investigation.

My dissertation is focused on physiological responses to cold stress in two drosophilid fly species with contrasting cold resistance: *Drosophila (Sophophora) melanogaster,* species of tropical origin with relatively low tolerance to cold stress, and *Chymomyza costata*, holarctic species with highly freeze-tolerant larval stage. So far, the insect cold-resistance literature has been dealing primarily with adaptations linked to gradual acquisition of cold resistance during entry into diapause and cold acclimation, meaning *prior to* cold stress. My dissertation, however, primarily explores the physiological processes related to repair of cold injury, meaning *after* the cold stress.

2. Insects at low temperatures

2.1. Definitions of low temperature

Insects living in habitats with pronounced seasonality are obliged to adjust their phenotypes accordingly. Many insects are polyvoltine, which means they produce several generations each year. Physiology of successive generations may profoundly differ. Therefore, it is absolutely essential to distinguish between summer and winter generations/phenotypes when performing experiments on insect cold resistance. The 'summer' generations/phenotypes are typically characterized by high behavioral and metabolic activity, growth, development and reproduction. Such processes must proceed at relatively high or close-to-optimum temperatures, otherwise they fail, which causes organismal death or, at least, loss of fitness. The 'winter' phenotypes are, on the other hand, characterized by dormancy, behavioral inactivity, cessation of growth and development - maintenance of basic cellular homeostatic processes only. This allows them to either operate at much lower body temperatures or even to go down to an absolute state of ametabolism (cryobiosis, anhydrobiosis). Dormancy together with multitude of specific adaptive changes produce cold resistant winter phenotype. Therefore, definitions of 'low' temperature will profoundly differ for summer and winter phenotypes of the same species.

Summer phenotypes of insects perform best at certain optimum temperature. In *Drosophila melanogaster*, for instance, such optimum is approximately at 25°C with highest fertility and fecundity of the adults (Klepsatel et al., 2019). With decreasing body temperature, the rates of biochemical and life processes slows down leading to developmental delay and to gradual loss of fitness. When the temperature reaches a lower developmental threshold (**LDT**) the development stops (the LDT in *D. melanogaster* is approximately at 11-13°C (Loeb and Northrop, 1917; Bliss, 1926). Hence, any temperature below LDT can be considered as 'low' for summer phenotypes. Although the insects do not die, they can live at temperatures corresponding to or slightly below LDT for only a limited period of time (days, weeks), mainly because they are usually unable to effectively search for food and their energy reserves will limit their survival time. Further decrease of body temperature to critical minimum (**CTmin**) will cause loss of neuromusculatory functions and cessation of all movements. i.e. onset of chill coma (Andersen and Overgaard, 2019) (the CTmin in *D. melanogaster* is approximately at 6-8°C, (Salachan and Sørensen, 2017)). When reaching the CTmin, the insect is sometimes considered as 'ecologically dead' because it cannot continue in foraging, mating and reproduction, or escape from predators. Physiologically, however, the insect is still alive. Relatively short (minutes, hours)

and mild excursions below CTmin are reversible, although sometimes paid by partial loss of fitness. Hence, the CTmin is often understood as lower lethal temperature (**LLT**) for summer phenotypes (Andersen et al., 2015). It has been shown for various insects that one of the primary causes of death in summer phenotype-insects exposed to temperatures below CTmin (LLT) is the inability to maintain osmotic and ion balance resulting in hyperkalemia, dissipation of transmembrane ion gradients, and unregulated opening of calcium channels leading to cell death and systemic collapse of whole organism (Koštál et al., 2004b; Koštál et al., 2006; Koštál et al., 2007; MacMillan and Sinclair, 2011b; MacMillan, 2013; MacMillan et al., 2015a; MacMillan et al., 2015c; MacMillan et al., 2016b; Bayley et al., 2018). The exact estimation of LLT in experimental conditions, however, is highly influenced by the time of exposure to low temperature. The larvae of *D. melanogaster* represent well the summer phenotype as they can occur in nature exclusively during warm part of season. When the larvae are acclimated at constant 15°C in the laboratory they will survive when exposed to: 6°C for couple of months; 5°C for approximately 1 day; and 0°C for only 3 h. Moreover, their LLT will strongly depend on acclimation state. The larvae acclimated at constant 25°C will die at 0°C within 10 min; the larvae acclimated at 15°C will die at the same temperature after 3 h, and the larvae acclimated at 6°C will survive at 0°C for 3.6 days (LLt50 according to (Koštál et al., 2011a)). These results document a general rule that the LLT is a highly plastic threshold. The LLT is considerably influenced by biologically meaningful variation (sex, age, developmental stage, feeding and health status, acclimation prior to stress) and also by experimental conditions (time of exposure, ramping rates, moisture, etc.). In the following text, I will use the term **cold tolerance** when speaking about low temperature limits in summer phenotype insects.

Winter phenotypes of insect species adapted to temperate or subpolar seasonality principally differ from summer phenotypes as they are linked to entry into a dormant state – either **quiescence** (*e.g.* adults of *D. melanogaster*) or **diapause** *(e.g.* larvae of *C. costata*) (see Chapter 2.2). Then LDT or CTmin thresholds lose their relevance because such insects *do not* develop and usually *do not* move nor show other behavioral activities. At the same time, other thresholds can be considered such as: melting point (**m.p.**) of body fluids; temperature of spontaneous ice crystallization or supercooling point (**SCP**); temperature of glass transition (**Tg**); etc. Most importantly, **LLT** of winter phenotypes is lowered in comparison to summer phenotypes, usually far below 0°C, and sometimes no LLT can be measured experimentally. Such high level of tolerance deserves a special term. I will use the term **cold hardiness** in following text when speaking about low

temperature limits in winter phenotypes of insects adapted to temperate and subpolar seasonality.

2.2. Winter phenotype

2.2.1. Diapause

Most insect species inhabiting temperate and polar regions have evolved life-cycles that are closely adapted to seasonality. Active summer phenotypes alternate with dormant winter phenotypes. **Diapause** is a systemically regulated form of dormancy is induced *prior to* the advent of environmental adversity, in response to changing environmental signals (most often photoperiod and thermoperiod). In contrast, **quiescence** is a form of dormancy occurring as a *direct response* to any environmental factor that actually decreases below a certain critical threshold (low temperature, high temperature, absence of food, lack of water) (Lees, 1955; Danks, 1987; Koštál, 2006). Diapause can be induced only at a very specific ontogenetic stage that is sensitive to environmental signals. In contrast, any developmental stage of any insect can, at least in theory, enter into low temperature-induced quiescence.

Diapausing insects are not locked in a 'static' state but they rather undergo a 'dynamic' succession of endogenously and exogenously driven changes in physiology ('physiogenesis' or 'phases of diapause development') (Andrewartha, 1952; Koštál, 2006; Koštál et al., 2017). During the *induction* phase, changes in photoperiod and thermoperiod are perceived by specific ontogenetic stages. For most insects, shortening of photoperiod after the summer solstice (short-day signal) indicates that deterioration of ambient conditions will come in future (upcoming decrease of ambient temperature during autumn). Therefore, the short-day signal induces a deep phenotypic transformation. The specialized photoperiodic receptors localized in insect brain and/or eyes (Numata et al., 1997) transduce the short-day signal into a change in synthesis and secretion of developmental hormones, ecdysone and juvenile hormone (JH) (Walker and Denlinger, 1980; Denlinger, 2002). Depending on ontogenetic stage at which diapause is overtly expressed, the decreasing/absent signaling by ecdysone (larval and pupal diapauses) or JH (adult diapauses) will systemically govern the phenotypic transformation in all peripheral organs. During *preparation* phase, the insects continue in intensive foraging activity in order to buildup energy reserves for upcoming harsh period (Hahn and Denlinger, 2007). At the same time, however, the proliferation activity of their gonads ceases (in the adults: males do not produce sperm, females stop oogenesis) and, consequently, all behavioral activities linked to sexual partner location, mating, and parental care are abolished. In the immature stages, the proliferation

activity in precursor cells (embryonic cells or imaginal discs in larvae) ceases (Koštál et al., 2009) and, consequently, the morphogenetic development is halted. This way, diapause starts and it is overtly expressed. During the early *initiation* phase of diapause, the locomotion and metabolic activity may still be very high. Many insects migrate for short distances to seek for overwintering microhabitats. Some species can even migrate at continental scales (Solensky, 2004). High metabolic activity linked to initiation phase is also needed to stimulate various protective mechanisms including, for instance, building cocoons (Danks, 1987), increasing the thickness of cuticular hydrocarbon layers (Benoit, 2010), upregulation of heat shock proteins (Rinehart et al., 2001), stimulation of antioxidant mechanisms (Jovanović-Galović et al., 2007), and inhibition of pro-apoptotic pathways (Villeneuve et al., 2006). The transcription profiles, protein expression patterns, and metabolic pathways also undergo deep restructuring (Emerson et al., 2010; Kankare et al., 2010; MacRae, 2010; Ragland et al., 2010; Poelchau et al., 2013; Poupardin et al., 2015; Yocum et al., 2015; Koštál et al., 2017). While the major task for metabolism in summer phenotype was to support high behavioral activity, growth and development by high rates of energy turnover, the task in winter phenotype will be to support relatively low rate of energy turnover for basal cellular metabolism and for maintenance of protective mechanisms. The next *maintenance* phase of diapause is characterized by very low activity and deep metabolic suppression despite the ambient conditions might be still fully permissive for high activity (end of summer, warm part of autumn). The ecological meaning of maintenance phase is to avoid prematurely reaching the next ontogenetic stage which would not be able to survive over winter. The gradual drop of ambient temperatures during autumn has two important consequences: first, diapause is gradually terminated and second, cold-acclimation processes are induced. The *termination* phase of diapause typically requires weeks or months of exposure to relatively low temperature before the sensitivity to photoperiodic signal is lost and a full potential to rapid resumption of development is returned. When this happens, the endogenously-imposed diapause reaches its end (diapause has been terminated). Because this happens during December/January in most insect species, the developmental potential cannot be realized but the insect will continue in exogenously-imposed dormancy – low temperature-quiescence. The post-diapausing quiescent insects wait for spring rise of temperature that will allow rapid and synchronous resumption of their behavioral activities and reproduction/development.

2.2.2. Cold acclimation, cold stress and recovery

The linkage of two adaptive complexes, diapause and cold hardiness, is a matter of debate (Denlinger, 1991). In most temperate insects, the cold hardiness increases already with the entry into diapause but the major seasonal increase of cold hardiness occurs only later, during the gradual fall of ambient temperatures in autumn (cold acclimation) and, therefore, coincides in time with the termination phase of diapause (Šlachta et al., 2002). Similarly as in the case of entry into diapause, the process of cold acclimation is associated with deep restructuring of transcriptome, proteome and metabolic pathways (Qin et al., 2005; Sinclair et al., 2007; Zhang et al., 2011; Colinet et al., 2013; Shang et al., 2015; MacMillan et al., 2016a; Koštál et al., 2017). I will discuss these restructurings in more detail in chapter 2.3.2.

As I have already argued in the Introduction, the rich insect cold hardiness literature was dealing for most of its 100-year-long history (Bachmetiev, 1907; Payne, 1927; Salt, 1961; Asahina, 1970; Zachariassen, 1985; Storey and Storey, 1988; Block et al., 1990; Lee and Denlinger, 1991; Sømme, 1995; Teets and Denlinger, 2013; Toxopeus and Sinclair, 2018) mainly with the phenotypic changes linked to cold acclimation – putative adaptive mechanisms, at many different levels of biological organization, potentially increasing cold hardiness *prior to* the stress exposure. In my dissertation, I attempted to broaden this view by analyzing the ultimate causes of cold and freezing injury at the cellular and subcellular levels (i.e. what happens *during* the stress exposure) and also studying the mechanisms linked to recovery *after* the stress exposure at the physiological, biochemical and gene expression levels.

3. Physiological mechanisms of insect cold hardiness

In this chapter, I will deal mostly with the cold hardiness *sensu stricto*, *i.e.* the adaptive complex associated with winter phenotypes of insect species inhabiting seasonal temperate and subpolar habitats. The insect cold hardiness literature recognizes three major adaptive complexes or 'strategies' of cold hardiness that are named according to what happens to body water at low temperatures, *i.e.* below the melting point of body fluids. The m.p. is dictated physically by osmolarity: each mol of dissolved solvent decreases the m.p. of a solution by 1.86°C (Zachariassen, 1985). For most terrestrial organisms, including insects and humans, the m.p. lays close to -0.5°C corresponding to osmolality of body fluids of 269 mOsm. When insect body temperature drops below m.p., three options exist for body water: (1) body water remains in liquid phase; (2) body water transits to solid phase, ice crystals; and (3) water molecules leave insect body in

vapor phase and they are deposited to surrounding ice crystals. Accordingly, three major strategies are: (1) **supercooling** or freeze avoidance; (2) **freeze tolerance**, and (3) **cryoprotective dehydration** (Zachariassen, 1985; Holmstrup and Westh, 1994; Holmstrup et al., 2002; Renault et al., 2002; Sinclair et al., 2003; Sinclair and Renault, 2010). Additionally, the small volume of remaining liquid solution after the freezing or after the evaporative dehydration may undergo another phase transition to amorphous solid (glass) phase, *i.e.* vitrification (Sformo et al., 2010; Koštál et al., 2011b).

It is important to stress that insect cold hardiness is currently understood as a complex adaptive syndrome composed of multiple components activated or stimulated during entry into diapause and cold acclimation (Lee, 2010; Teets and Denlinger, 2013; Toxopeus and Sinclair, 2018). The roles of different components may differ in different strategies. I will briefly discuss major adaptive components and I will also compare their possible roles in two most often explored strategies, supercooling and freezing with occasional notes on the third strategy, cryoprotective dehydration.

3.1. Water balance, cuticle

Supercooled overwintering insects retain almost full volume of body water and need to protect this pool for long time (months) often in a situation with no possibility for replenishing (immobile diapausing stages such as embryo, prepupa, pupa). It has been shown that some insects change the composition of cuticular hydrocarbons and/or increase the thickness of their cuticle during entry into diapause in order to minimize the respiratory loss of water (Yoder et al., 1992; Kaneko and Katagiri, 2004; Yocum et al., 2009; Benoit, 2010; Ala-Honkola et al., 2020). The respiratory loss of water is reduced by metabolic suppression innate to diapause and also by direct effect of low temperature on the rate of metabolism. In addition, many insects, including the diapausing stages, can reduce the respiratory loss of water by employing the strategy of discontinuous respiration when their spiracula open for just short intervals separated by long periods when they are closed (Chown and Davis, 2003).

Insects relying on cryoprotective dehydration, in contrast, keep their cuticle highly permeable for water. Since the water vapor pressure above the pool of supercooled water inside the insect body is *higher* than the water vapor pressure above the surrounding ice crystals (snow, ice, frozen water in soil pores, etc.), the water molecules easily and rapidly escape from small insects body through permeable cuticle and, later, join the ice crystals in environment (Holmstrup and Westh, 1994; Holmstrup et al., 2002). This way, insects may lose most of their

body water which renders the remaining body solutions highly concentrated so that they cannot freeze. On the other side, the cells are exposed to all deleterious effects linked to loss of water activity.

Freeze-tolerant insects survive after formation of ice crystals inside their body. Intracellular formation of ice is considered almost invariably lethal (but some exceptions do exist (Worland et al., 2004; Sinclair and Renault, 2010)). Two basic mechanisms ensure that extracellular freezing occurs at relatively high temperatures (close to m.p.): (i) a cuticle that is highly permeable for surrounding ice crystals will allow penetration of ice into the body cavities and inoculative freezing of body fluids (Rozsypal, 2015; Rozsypal et al., 2018); (ii) cytosol or cell interior usually do not contain any potent nucleators of ice. In order to actively initiate ice nucleation in extracellular fluids, some insects seasonally secret specific protein ice nucleators (PINs, will be discussed later) into hemolymph (Zachariassen, 1985). As the water vapor pressures above the frozen body fluids and surrounding ice equalize, the freeze tolerance is considered as water-saving strategy. On the other side, however, the cells are again exposed to all deleterious effects linked to loss of liquid water activity, similarly as in the case of cryoprotective dehydration.

The ratio of osmotically active ('bulk') and osmotically inactive ('bound') water can change, at least in theory, in summer and winter phenotypes (Block, 2002). Part of the total pool of water molecules is always non-covalently bound in the hydrations shells of ions, small charged molecules, and diverse macromolecules (Franks, 1986). Since some macromolecules are characteristically accumulated for diapause (glycogen, storage proteins) in winter phenotypes, they can 'bind' more water molecules. Similarly, winter phenotypes often accumulate large amounts of low molecular mass cryoprotectants (CPs, see chapter 3.7.) and they also 'bind' water molecules. The bound water molecules are relatively immobile and not easily available for ice formation. Hence, in freeze-tolerant insects the increasing water binding capacity would reduce the amount of ice generated at a given low temperature and, this way, would increase the cold hardiness (Block, 2002; Rozsypal et al., 2018).

3.2. Osmotic and ionic homeostasis

I have stressed in chapter 2.1. the importance of maintaining regulated water and osmotic balance for summer phenotypes of any insect in order to survive at relatively low temperatures below LDT or CTmin but still above zero or m.p. of body fluids (MacMillan and Sinclair, 2011a; MacMillan and Sinclair, 2011b; MacMillan et al., 2015a; Gerber and Overgaard, 2018). The same principle holds

true also for supercooled winter phenotypes of adapted insects. It has been shown that supercooled insects do maintain transmembrane electrochemical potentials (*i.e.* ion gradients) (Koštál et al., 2004b; Zachariassen et al., 2004). It is not entirely clear, however, how they do so. Increasing the activity of a primary ion pumping system, Na^+K^+ ATPase, can hardly be the solution for two reasons: low temperature directly prevents high enzymatic activity and the energy resources would not suffice to supply ATP for very long period of overwintering. Most likely, the solution is complex, taken at the systemic level, and including the regulation and closing ('channel arrest' theory) of a variety of ion pumping systems and ion channels in different tissues involved in osmoregulation such as Malpighian tubes and rectum (Hochachka, 1986; MacMillan and Sinclair, 2011b; MacMillan et al., 2015a).

Freeze-tolerant insects, in contrast, dissipate transmembrane electrochemical potentials during internal freezing and a situation close to Donnan's equilibrium is established (Kristiansen and Zachariassen, 2001). Of course, the ion gradients need to be reconstituted soon after melting and cellular rehydration (Kristiansen and Zachariassen, 2001; Štětina et al., 2018).

3.3. Metabolic suppression and regulation of pathways

Cold acclimation is inevitably associated with metabolic suppression due to the direct effect of temperature on the enzyme kinetics and the rate of biological processes. The metabolic suppression during diapause and cold acclimation is often deeper than that expected based on application of simple Q_{10} rule (Hochachka, 1986). This is because many processes are completely inhibited, rather than slowed down. The supercooled insects need to cope with limited stability and life-span of proteins in liquid environment. This most likely requires slow continuation of processes such as gene transcription and protein turnover. Supercooled insects also need to maintain basal cellular homeostasis, including the transmembrane ion gradients (Koštál et al., 2004b). Such processes require access to energy in the form of ATP and, therefore, supercooling requires continuity of regulated metabolism. Frozen insects, in contrast, can save energy as they most likely do not continue in regulated metabolism. Insects frozen at ecologically relevant temperatures, however, usually are not cryopreserved in a static state (Toxopeus and Sinclair, 2018), but still show signs of anaerobic metabolism, such as slow accumulation of lactate (Storey and Storey, 1985).

Some specific metabolic pathways are paradoxically upregulated at low temperatures. The stimulation of glycogen degradation at temperatures below +5°C is the best known example (Storey and Storey, 1991). The glucose released from

glycogen is directed toward synthesis and accumulation of various CPs, such as glycerol, sorbitol, and ribitol. The reduction of sugars to corresponding polyols requires access to reducing power in the form of NADPH produced mainly in pentose cycle, which also needs to be upregulated (Storey and Storey, 1991; Koštál et al., 2004a).

3.4. Remodeling of biological membranes

Entry into diapause and cold acclimation are associated with restructuring of the composition of biological membranes (Sinensky, 1974; Cossins, 1994; Kostal, 2010). Several changes are stereotypically observed in different species of plants and animals, including insects: (i) the increasing desaturation of fatty acyls; (ii) the shortening of fatty acyl chain lengths; (iii) the increase of relative proportion of phosphoethanolamines at the expense of phosphocholines; (iv) the change in relative proportions of sterols (Cossins, 1994; Hazel, 1995; Koštál et al., 2013). This restructuring cause change of biophysical properties of membranes and, this way, contributes to functionality and integrity of membranes in supercooled and frozen insects (McElhaney, 1984; Hazel, 1995). The membranes of supercooled insects need to maintain specific fluidity required for functionality of embedded proteins (including the ion transport systems). The supercooled insects also need to prevent unregulated transition from liquid-crystalline to gel phase, which would result in membrane leakiness. The frozen insects, in contrast, need not to keep their membranes functional but need to protect their integrity during insults linked to growth of extracellular ice crystals and severe cell dehydration. Dehydrated phospholipid bilayer rich in ethanolamine (a situation typical for insects) is prone to unregulated transition to hexagonal phase, which would mean complete loss of membrane barrier function. Closely packed membranes (a situation resulting from cellular dehydration during extracellular freezing), are also prone to fusions (Uemura et al., 1996; Hincha et al., 1998).

3.5. Bolstering of cellular protective systems

During early phases of diapause and/or gradual cold acclimation, the insects adjust basic cellular protective systems to winter phenotype. For instance, both supercooling and freeze-tolerant insects upregulate specific heat shock proteins (HSPs) (Rinehart et al., 2006; King and MacRae, 2015; Toxopeus et al., 2019a). Rapid expression of inducible HSPs, however, is also one of the most obvious events linked to recovery after cold stress (Goto and Kimura, 1998; Sinclair et al., 2007; Koštál and Tollarová-Borovanská, 2009; Štětina et al., 2015). The HSPs can stabilize protein complexes such as cytoskeleton (Russotti et al., 1997; Des

Marteaux et al., 2018a) and/or they can bind hydrophobic domains exposed in partially denatured or misfolded proteins occurring in higher abundances after cold or freeze-dehydration stress. After binding to HSPs, the damaged proteins are either actively refolded or they are directed toward proteasome degradation pathway (Parcellier et al., 2003; Strudwick and Schröder, 2007).

The oxidative stress might be an important cause of injury linked with cold or freezing stress in insects (Rojas and Leopold, 1996; Lalouette et al., 2011; Torson et al., 2015). Therefore, it is reasonable to expect that various enzymatic and non-enzymatic systems for coping with oxygen radial species will be upregulated in preparation for potential cold stress (either during entry into diapause and/or during cold acclimation).

3.6. Macromolecular cryoprotectants

Two classes of proteins associated with regulation of ice nucleation within insect body are specifically accumulated by insects in preparation of winter season: antifreeze proteins (AFPs) and protein ice nucleators (PINs) (Zachariassen and Kristiansen, 2000; Duman, 2001; Duman et al., 2010; Duman, 2015). The supercooled insects sometimes employ the AFPs that adsorb on the surface of ice nucleus and suppress its growth by preventing the water molecules to join the ice crystal. This way, the temperature of ice crystallization (freezing) is decreased to approximately -2°C to -8°C and the thermodynamically unstable supercooled phase of water is stabilized. Paradoxically, AFPs are active also in some freeze-tolerant insects, which prefer to freeze at relatively high subzero temperatures. In these insects, the AFPs usually show only 'weak' antifreeze activity (suppressing the freezing temperature by only a fraction of °C), and they rather serve a different function: to prevent ice recrystallization (Capicciotti et al., 2013).

PINs, in contrast to AFPs, stimulate ice crystallization at relatively high subzero temperatures. PINs expose a 'surface' domain which can catalyse organization of water molecules into the ice crystal lattice. This way, PINs prevent unwanted supercooling in freeze-tolerant insects and ice crystals to emerge in desired body compartments (Zachariassen and Kristiansen, 2000).

HSPs are proteins with chaperone function and their general functions are already mentioned in Chapter 3.5. The HSPs are evolutionary conserved with highly similar sequences found across many prokaryotes and eurkaryotes (Plesofsky-Vig, 1996; Kiang and Tsokos, 1998). There are two major classes of inducible HSPs in *D. melanogaster*, with close homologues in other animal species – class of 70 kDa HSPs and small HSPs (e.g. Hsp22, Hsp23, Hsp26, Hsp27) (Lindquist and Craig, 1988; Korsloot et al., 2004). Inducible HSPs are

expressed after stress. Originally, massive up-regulation of HSPs was discovered in response to heat stress (Burdon, 1986). Similar responses, however, were later observed following cold stress and number of other challenges of physical, chemical, and biological nature. In insects, the cold-associated upregulations of HSPs were observed either during cold acclimation or during recovery after cold stress (Burton et al., 1988; Goto and Kimura, 1998; Nielsen et al., 2005; Qin et al., 2005; Sinclair et al., 2007; Colinet et al., 2010b; Udaka et al., 2010; Štětina et al., 2015). The often observed massive increase of Hsp70 mRNA levels led to hasty interpretations of its role in the repair of cold injury. Direct functional analyses in *Drosophila melanogaster* and other insects, however, brought either limited or no support for such interpretations. In our study (Štětina et al., 2015), we analyzed the expression levels of 24 different mRNA transcripts of the HSPs complex in response to cold in two strains of *D. melanogaster*: the wild-type and the *hsp70*- null mutants lacking all six copies of *hsp70* gene. The larvae of both strains showed similar patterns of HSPs complex gene expression. No transcriptional compensation for missing *hsp70* gene was seen in the mutant strain and no differences in mortality were found between two strains when the larvae were exposed to relatively mild doses of cold. However, the cold tolerance in *hsp70*-mutants was clearly impaired when the larvae were exposed to severe and acute cold shocks. In agreement with the conclusions earlier formulated by Nielsen et al. (2005), our results suggest that the cold-induced up-regulation of Hsp70 need not necessary for repair of cold injury in all situations.

A particular group of drought stress-induced proteins was repeatedly observed in different eukaryotic organisms – so called LEA proteins. Originally discovered in plants (Cuming and Lane, 1979), LEA proteins were identified as important part of mechanism allowing osmoprotection in desiccated seeds. Since the first discovery, similar proteins were found in fungi, algae, and microbes, as well as in anhydrobiotic animals such as protozoa, nematodes, insects, and crustaceans (Shih et al., 2008). The larvae of chironomid fly *Polypedilum vanderplanki* inhabit shallow rock pools prone to drying in central Africa. The larvae can survive almost complete dehydration and the specific LEA proteins most probably act as molecular shields during this stress, preventing protein denaturation and aggregation (Goyal et al., 2005; Hand et al., 2011). Hence, LEA proteins might also be associated with survival of low freezing stress, which is always inevitably associated with cellular dehydration (see Chapter 3.1). Delicate structures inside dehydrated cell come to close proximity (crowding) and some protective mechanisms are expected to be present. So far no studies on the role of LEA proteins in freeze-tolerant insects were conducted.

3.7. Low molecular mass cryoprotectants (CPs)

Different organisms can accumulate small protective molecules in response to environmental stress (Yancey, 2005). These molecules are often called compatible osmolytes, as they were first described in organisms coping with osmotic stress. Later, however, similar compounds were observed in most forms of life, and were found to have a variety of protective functions (Yancey and Siebenaller, 2015). The accumulation of small molecules is also one of the hallmarks of insect adaptation to cold (Salt, 1961; Sømme, 1982; Zachariassen, 1985; Lee, 2010). The cryoprotectants (CPs) are most often represented by sugars and polyols (Storey and Storey, 1988; Storey and Storey, 1991) and by free amino acids (Koštál et al., 2011b). The specific roles of CPs may vary according to cold-tolerance (Storey and Storey, 1988). Generally, the CPs can act by one of the three broad mechanisms: (i) via affecting the water phase behavior; (ii) via metabolic protection (antioxidation, redox balance maintenance, detoxification); and (iii) via stabilization of macromolecules, which is based on specific interactions between the solvent (water), solutes (CPs) and a protected structure (protein, nucleic acid, lipid bilayer).

Ad (i): Relatively high concentrations of accumulated CPs will colligatively suppress the m.p. of biological solutions and, correspondingly suppress also the temperature of SCP (Zachariassen, 1985). This way, the metastable supercooled phase of water is stabilized and supercooling insects can 'avoid' lethal freezing event. The concentrations of accumulated CPs can reach 'astronomic' values of 3M (*Ips acuminatus*,(Gehrken, 1984) or even 5M *(Bracon cephii*, (Salt, 1961)). Such high concentrations make the biological solution virtually unfreezable under ecological conditions. In freeze-tolerant insects, the accumulation of very high concentrations of CPs would be counterproductive, as these insects 'prefer' to freeze at relatively high subzero temperatures, nevertheless many freeze-tolerant insects were found to accumulate low to medium concentrations of various CPs (Storey and Storey, 1988). In this case, the general role of accumulated CPs is to reduce the amount of ice generated at any given subzero temperature and, this ways, to reduce the osmotic outflow of water from cells, *i.e.* reduce the cell shrinkage (Meryman, 1971; Storey and Storey, 1988; Rozsypal and Košťál, 2018; Rozsypal et al., 2018). In addition, the remaining highly concentrated solutions may transit into amorphous glass phase in insects that underwent cryoprotective dehydration or freeze-induced cellular dehydration, (Rudolph and Crowe, 1986). It has been show in at least two insect species that this really happens and, moreover, that the glass transition could be adaptive (Sformo et al., 2010; Koštál et al., 2011b; Rozsypal et al., 2018).

Ad (ii): Many CPs have antioxidant capacity (for instance trehalose, (Reyes-DelaTorre et al., 2012); and proline, (Kaul et al., 2008)) can, at least in theory, protect the overwintering insects against oxidative damage. The biosynthesis of polyols from sugar precursors consumes reducing power in the form of NADPH. As the NADPH is also required for re-reduction of oxidized glutathione, the metabolism of polyols may affect the redox balance.

Ad (iii): Both, low temperature and low activity of water directly threaten stability of macromolecules and integrity of macromolecular complexes. Number of polymeric proteins will depolymerize already at moderately low temperatures around zero (Privalov, 1990). Despite depolymerized proteins lose their activity (enzymes, signaling molecules) or structural function (cytoskeleton), the depolymerization is usually reversible upon return of normal conditions and, therefore, need not negatively affect the viability of cold stressed insects (Des Marteaux et al., 2018b). Proteins may also denature (*i.e.* exposed their internal hydrophobic domains) in liquid environment at very low temperatures (supercooled state). At least in theory, the occurrence of cold and heat denaturation of proteins is similarly probable at extremely low and high, respectively, temperatures (Dias et al., 2010; Sanfelice and Temussi, 2016). Denatured proteins may aggregate (*i.e.* tightly interact via their 'naked' hydrophobic regions). As the aggregation is hardly reversible, it will negatively affect the viability of an insect after cold stress. Phospholipid bilayers undergo phase transition to non-functional gel phase at low temperatures (see chapter 3.4.). In supercooled insects, the accumulated CPs can protect native protein structures as well as functional liquid crystalline bilayer phase via a mechanism know as *preferential exclusion* (Arakawa and Timasheff, 1985; Timasheff, 1992). In aquatic solutions, many CPs behave as *kosmotropes*, *i.e.* compounds highly soluble in water, preferring interactions with water molecules over interactions with proteins or phospholipid headgroups. Consequently, kosmotropic CPs are preferentially excluded from macromolecular vicinities, rendering the macromolecules preferentially hydrated and thermodynamically more stable (Xie and Timasheff, 1997; Timasheff, 1998; Jensen et al., 2004; Ball, 2008).

Cells of freeze-tolerant insects are exposed to low temperatures in combination with two other potent stressors: almost absolute absence of liquid bulk water and presence of ice crystals. Therefore, freezing stress is potentially more risky than just supercooling to the same temperature. The stressors linked with freezing and freeze-induced cellular dehydration affect four levels of biological organization simultaneously: (i) at a molecular level, they cause decrease of kinetic energy and reactivity of all molecules, including enzymes, signaling cascades, etc.

Molecular mobility is reduced and, thus, the functional conformation, stability of macromolecules and integrity of their complexes are severely threatened. (Carpenter and Crowe, 1988; Franks and Hatley, 1991; Muldrew et al., 2004; Des Marteaux et al., 2018a). Nucleic acids may change conformation from biologically relevant B-form to nonfunctional A-form at low water activity (Brovchenko and Oleinikova, 2008), and the exposed loops of euchromatin DNA may physically break when mechanically stressed (Lubawy et al., 2019); (ii) impaired molecular function affects the metabolic level. The biochemical and cellular processes are strongly inhibited. The 'disordered' metabolic conversions may cause gradual depletion of energy and/or buildup of toxic intermediates (Storey and Storey, 1985; Joanisse and Storey, 1996); (iii) at cellular and tissue levels, the osmotic fluxes of water (out of the cell during extracellular freezing / into the cell during melting) and growing ice crystals exert osmotic and direct mechanical stresses, respectively, on delicate structures of the organelles, cell, and extracellular matrix. The cells and tissues may lose their physical integrity (Lovelock, 1954; Mazur, 1984); (iv) at a systemic level, injured molecules, structures, cells and organs may fail to interact properly during repair of freezing injury and resumption of life functions after freezing stress, causing organismal collapse or loss of fitness (Pörtner, 2002; MacMillan, 2019). Considering the formidable combination of deleterious effects linked to internal freezing, it is rather surprising to see that freeze tolerance has evolved independently and repeatedly in some ectotherm vertebrates (Storey and Storey, 1992), and in many invertebrates and insects (Salt, 1961; Asahina, 1970; Storey and Storey, 1988; Chown and Sinclair, 2010).

High concentrations of specific CPs are believed to protect native protein structures against freeze dehydration-induced denaturation and aggregation (Inoue and Timasheff, 1972; Tamiya et al., 1985; Franks and Hatley, 1991; Wang, 1999; Bolen and Baskakov, 2001; Kaushik and Bhat, 2003; Toxopeus and Sinclair, 2018). Upon loss of water during cellular freeze dehydration, most of the bulk water is removed (as the molecules joined ice crystal lattice) and the cell interior becomes more viscous. Under such conditions, the mechanism of preferential exclusion fails to work. Most kosmotropic CPs are unable to protect proteins or phospholipid bilayers beyond the threshold hydration of 0.3 g water per gram of dry mass (Hoekstra et al., 2001). However, the trehalose and some other di- and oligo-saccharides were shown to have remarkable ability to stabilize membranes during desiccation (Crowe et al., 1984; Thompson, 2003). They can form hydrogen bonds with polar residues on phospholipid headgroups and, this way, replace the missing water molecules and stabilize the membrane in the absence of water. Hence, the mechanism is known as *water replacement* (Crowe et al., 2001; Crowe,

2007). Proline and arginine are very specific among all amino acids in their ability to *directly interact* with partially denatured proteins (resulting from freezing stress) and stabilize them in a molted globule phase, while preventing their further denaturation and aggregation (Samuel et al., 1997; Das et al., 2007; Lange and Rudolph, 2009; Schneider et al., 2011). Proline and arginine are also the only two amino acids that display, at high concentrations and low water activities, a high propensity to self-associate and form supramolecular clusters (*stacked columns*) displaying hydrophobic surface (aligned methylene groups), which interact with hydrophobic surfaces of partially unfolded proteins (Rudolph and Crowe, 1986; Das et al., 2007). Perhaps not surprisingly, proline and arginine have been found as two most potent cryoprotective compounds in a large screening bioassay of 31 different native amino acids in *D. melanogaster* (Koštál et al., 2016a). Moreover, high concentrations of proline alter temperature of bilayer phase transitions and reduce tendency for fusion in closely packed membranes through hydrophobic interactions of proline imino-groups with the hydrocarbon chains of the bilayer phospholipids (Anchordoguy et al., 1987).

Another deleterious consequence of cellular freeze dehydration is that cytoplasmic components become crowded, organelles come into close contact, and their membranes may fuse (Uemura et al., 1996). It has been proposed that a wide selection of CPs may serve in this situation as sort of *molecular shield*, preventing unwanted interaction between tightly adjacent phosholipid bilayers or even proteins (Bryant et al., 2001; Hoekstra et al., 2001; Ball, 2008).

Finally, high concentrations of CPs, such as trehalose of proline, were shown to stimulate transition of highly concentrated body solutions into the amorphous phase (Rudolph and Crowe, 1986) This glassy phase may further stabilize all structures and protect them against thermomechanical stress (Rubinsky et al., 1980).

3.8. Cold acclimation vs. recovery after cold stress

It is obvious from the above list of mechanisms that most of them are associated with the entry into diapause and/or cold acclimation. They are stimulated *prior to cold stress*, and they are believed to *prevent* occurrence of cold injury; *i.e.* to promote the *resistance* against cold injury.

My dissertation, in contrast, focuses on events that occur *after the cold stress*. I deal with mechanisms that may serve to *tolerate* or *repair* the injury that potentially occurred during the cold or freezing stress.

4. Model insects

My PhD project was based on comparative analysis of two insect models with contrasting levels of cold tolerance or cold hardiness. On one side, a famous model of modern biology, *Drosophila* (*Sophophora*) *melanogaster* – a fly with tropical origin, currently spreading into temperate regions around the world, that exhibits relatively modest level of cold tolerance (Throckmorton, 1975; Kellermann et al., 2009; Strachan et al., 2011). On the other side, temperate and subpolar drosophilid fly, *Chymomyza costata* – one of the most cold-hardy organisms on the Earth and emerging model for investigation into extreme freeze tolerance (Moon et al., 1996; Koštál et al., 2011b; Des Marteaux et al., 2019).

The temperate populations of *D. melanogaster* overcome cold winter spells as adults in reproductive arrest (Saunders et al., 1990; Saunders and Gilbert, 1990). Low ambient temperature (below LDT at 11-13°C, (Bliss, 1926)) is the main trigger of ovarian dormancy, which indicates that the reproductive arrest is primarily a direct response to a limiting factor, *i.e. quiescence* rather than systemically regulated *diapause*. Nevertheless, the term 'diapause' is still used (wrongly, to my opinion) by many authors to describe the state of *D. melanogaster* adults overwintering in temperate regions (Williams and Sokolowski, 1993; Schmidt et al., 2005; Allen, 2007; Schmidt and Paaby, 2008; Kubrak et al., 2014). In contrast, larvae of *C. costata* respond to photoperiodic and thermoperiodic signals and enter classical diapause as fully grown third instar larvae (Riihimaa and Kimura, 1988; Yoshida and Kimura, 1995; Koštál et al., 2000; Koštál et al., 2016c).

All developmental stages of *D. melanogaster* (not only the adults in reproductive arrest) show some phenotypic plasticity in cold tolerance. It means that they can positively respond to cold acclimation by lowering the limits of CTmin and LLT (Czajka and Lee, 1990; Chen and Walker, 1994; Rajamohan and Sinclair, 2009). The cold acclimation of *D. melanogaster* is associated with physiological modifications resembling those typical for long-term, developmental acclimation of diapausing, cold-hardy insects. For instance, cold-acclimated adults of *D. melanogaster* restructured their biological membranes (Overgaard et al., 2008); expressed heat shock proteins after cold stress (Sinclair et al., 2007); changed metabolomic profile (Overgaard et al., 2007; Colinet et al., 2016), reorganized the transcriptome (MacMillan et al., 2016a), and protected their osmotic and ionic balances (Armstrong et al., 2012; MacMillan et al., 2015b). The cold-acclimated larvae of *D. melanogaster* showed cold-induced responses very similar to adults (Koštál et al., 2011a; Koštál et al., 2016b). I was specifically involved in analysis of the role of the Hsp70 in cold acclimation and cold tolerance of *D. melanogaster* larvae (Štětina et al., 2015). Using Hsp70 null mutants lacking

all six copies of the *hsp70* gene we obtained direct genetic proof for involvement of Hsp70 protein in repair of cold injury in *D. melanogaster* (see Chapter 3.6). Next, we found that a combination of cold-acclimation and feeding on proline-augmented diet results in *D. melanogaster* larvae in development of capacity to tolerate internal freezing at relatively moderate subzero temperature of -5°C (Koštál et al., 2012). Frozen larvae had 75-77% of their body water converted to ice, but they were still able to recover after melting, pupariated and produced viable adults (Rozsypal et al., 2018). Later, number of other aminoacids (mainly arginine, but also isoleucine, aspartate, alanine, valine, and leucine), and some related compounds (such as ectoine), were used as food additives and were shown to increase larval freeze-tolerance (Colinet et al., 2016). Induction of quiescence and larval cold acclimation appeared as potentially applicable strategy for long-term storage of *D. melanogaster*. While larval development takes only 2 days at 25°C, maintaining the cold-acclimated quiescent larvae at a specific thermal regime with fluctuating low temperatures (6°C for 20 h/11°C for 4 h) we were able to extend the larvae storage period for 2-3 months (Koštál et al., 2016b).

The larvae of *C. costata* show dramatic phenotypic plasticity in their cold hardiness trait, especially in the capacity to survive cryopreservation in liquid nitrogen (which we sometimes refer to as 'extreme freeze tolerance', *i.e.* no LLT for freeze tolerance seems to exist). The active, non-diapause, warm acclimated larvae are relatively freeze susceptible, they can tolerate freezing only at mild subzero temperatures (-5°C) while upon phenotypic change linked with the entry into diapause followed by cold acclimation, the larvae can survive deep freezing in liquid nitrogen (-196°C, LN_2) (Moon et al., 1996; Koštál et al., 2011b).Entry into diapause (Koštál et al., 2016c) results in cessation of development, loss of behavioral activities, and deep metabolic suppression (Koštál et al., 2000; Koštál et al., 2009), all based on massive alteration of gene expression (Poupardin et al., 2015; Koštál et al., 2017). Cold acclimation stimulates metabolic reorganization resulting in accumulation of free proline that reaches concentrations higher than 0.3 M (Rozsypal et al., 2018). Proline exogenously added to non-diapause larvae, via proline-augmented diet, increases their freeze tolerance and renders them even cryopreservable in LN_2 (Koštál et al., 2011b; Rozsypal et al., 2018). The cold acclimation is associated with further change in transcriptomic profile (Koštál et al., 2017) suggesting specifically enhanced capacity for protein folding, refolding and processing (Des Marteaux et al., 2019).

5. Goals of PhD project

During my PhD study, I have participaed in several side-projects, which was reflected in my co-authorship on four papers (Koštál et al., 2016b; Koštál et al., 2017; Des Marteaux et al., 2018b; Toxopeus et al., 2019b). I decided to include into my PhD dissertation other three papers where I am the first author and where my participation was significant, including not only practical experiments but also conceptual steps such as designing the experiments, development and establishment of methods, interpretation of results, and scientific writing. First two papers (Štětina et al., 2018; Štětina et al., 2019) represent direct answer to the major PhD project goal as formulated in the study plan (study on recovery after the cold stress). Third paper has been submitted for publication and it is now under revision. The topic of third paper emerged during the work on recovery after stress, which indicated that mitochondria might be important targets of cold- and freezing-induced injury in insects.

5.1. Recovery after cold stress

Physiological adjustments accompanying insect cold acclimation *prior to* cold stress have been relatively well explored (see chapter 3). In contrast, recovery from cold stress received much less attention. The first goal of my PhD project was to analyze the physiological processes, and changes in metabolomic and transcriptomic profiles, occurring after the cold stress of varying intensity in the larvae of two model insects, *D. melanogaster* and *C. costata*.

5.2 Mitochondria as targets of low temperature-induced injury

The second goal of my PhD project was to get insight into the role of mitochondria in response to freezing stress in larvae of *C. costata*. Although some knowledge has been accumulated on changes in mitochondrial counts and activity during entry into insect diapause, the responses of insect mitochondria to cold and freezing stress were minimally studied so far.

6. Main results of PhD project and their discussion

In this chapter, I will briefly comment on the results of three papers which are presented in their full form in Chapter 8.

6.1. Recovery from cold stress – *Chymomyza costata*

The results were published in the paper:

Štětina T., Hůla P., Moos M., Šimek P., Šmilauer P., Košťál V (2018). Recovery from supercooling, freezing, and cryopreservation stress in larvae of the drosophilid fly, *Chymomyza costata*. Scientific Reports 8, 4414 (see chapter 7.1) Electronic supplementary materials are available at https://doi.org/10.1038/s41598-018-22757-0

In this study, we aimed to drive the attention of scientific community in the field of insect cold hardiness toward the processes linked to recovery after cold stress. We argued that the insect cold hardiness should be considered as a holistic and sequential process of diapause induction, cold acclimation, cold injury, and the *repair or tolerance* of cold injury. While the first two elements, (*i.e.* diapause induction and cold acclimation) of the process received significant attention (see chapters 2. and 3.), the nature of injury and especially, the role of repair processed have been neglected so far. Some earlier results, however, suggest that active repair processes do participate in recovery after cold stress. For instance, the cold acclimation-induced upregulation of various cellular protective systems, such as those preventing apoptosis (Yi et al., 2007), oxidative damage (Storey and Storey, 2010; Lalouette et al., 2011), or protein denaturation (Sørensen et al., 2003), may represent a 'potentiation' step that is fully realized only *after* the stress. Specifically the activation of HSPs production after the cold stress was stereotypically observed in many insects (Goto and Kimura, 1998; Nielsen et al., 2005; Sinclair et al., 2007; Colinet et al., 2010b; Zhao and Jones, 2012; Des Marteaux et al., 2019), and direct genetic proofs for importance of HSPs expression in insect cold hardiness are available (Rinehart et al., 2007; Koštál and Tollarová-Borovanská, 2009; Colinet et al., 2010a; Štětina et al., 2015). Another hint pointing toward the active participation of repair processes in recovery after the cold stress comes from studies documenting so called 'delayed mortality'. Such mortality does not occur immediately during or after the stress but it is significantly delayed, by days or weeks, often to the later stages of ontogenesis (Bale et al., 1989; Yocum et al., 1994; Koštál et al., 2019). This delayed mortality might be regarded as an inability to *repair* any vital injury that occurred during previous exposure to cold.

In this study, we exposed the diapausing, cold-acclimated larvae of *C. costata* to three different levels of cold stress: supercooling to -10°C; freezing at -30°C, and cryopreservation at -196°C. Because the supercooled larvae almost do not suffer from any mortality, we expected to see practically no differences between supercooled and control larvae (the control larvae were not exposed to stress, but they were transferred from low acclimation temperature of 4°C to relatively high temperature of 18°C, which is permissive for resumption of development including the potential activation of repair processes). Next, we

expected to see clear differences between supercooled and frozen larvae, as the freezing stress in much more severe than just supercooling. Despite the severity of freezing stress, the larvae show very high survival and, therefore, we hoped to see clear signatures of active repair processes in their metabolomic and transcriptomic profiles. Furthermore, the cryopreservation represents the most stressful conditions resulting in delayed mortality in approximately 50% of larvae. Based on differences between frozen and cryopreserved larvae, we hoped to identify structures and processes vulnerable to injury caused by rapid changes of temperature during cryopreservation.

The results confirmed our expectations only partially. It was surprising to see that the metabolomic and transcriptomic profiles profoundly differed between control and supercooled larvae. This result suggested that even the extremely cold hardy larvae of *C. costata* are significantly affected (injured) by a mild cold stress (supercooling to -10°C), which causes practically no mortality. In fact, the metabolomic and transcriptomic profiles were closely similar in supercooled and frozen larvae, despite the profound difference in severity between cold and freezing stress (see chapter 3.1.). For instance, the supercooled larvae protect and maintain the ion gradients, while the frozen larvae dissipate them and need to recover them after the cold stress. We expected that this process might take some time and will require access to significant amount of energy in frozen larvae. The results, however, indicated that full reestablishment of ion homeostasis is finished within 1 h after melting, and both supercooled and frozen larvae consume more oxygen (by approximately 20%) than controls during 3-d-long recovery after the respective cold stress. Finally, the most significant differences in metabolomic and transcriptomic profiles were found between supercooled/frozen and cryopreserved larvae. Based on these differences, we were able to identify that impaired mitochondrial function might be the potential cause of cold and freezing injury in *C. costata* larvae (see Fig. 1): (i) The accumulations of glucose and several derivates of the glycolytic pathway side-branches, such as fructose, myo-inositol, sorbitol, and glycine, suggest that the glycolytic flux (liberated from glycogen and/or absorbed from diet) is partially diverted from TCA and production of energy toward accumulation of alanine. (ii) The catabolism of lactate and cysteine accumulated during the previous anaerobic episode might contribute to production of excess pyruvate, which again might be canalized preferentially to alanine rather than to TCA. (iii) The level of sarcosine (a derivative of glycine) has been reported to increase in cancer cells, known to divert their glycolytic flux from TCA to anaerobic end-products. (iv) The relatively low levels of citrate, aconitate, and ketoglutarate might result from partial blockade of pyruvate entry to, or early steps

of, the TCA cycle in the mitochondrial matrix [see (i) – (iii)]. (v) The accumulation of ornithine (the synthesis of which is also located inside mitochondria) might indicate re-routing of mitochondrial catabolic pathways for excess amino acids (proline, glutamine, glutamate) from TCA (partially blocked) toward ornithine.

At the same time, the polyamines (putrescine, spermine, and spermidine), to which ornithine can be further transformed upon decarboxylation in the cytosol, were undetectable or found in traces in all treatments. (vi) The last segment of the TCA cycle, catabolism of succinate accumulated during a previous anaerobic episode seems to operate normally in larvae cryopreserved at -196°C.

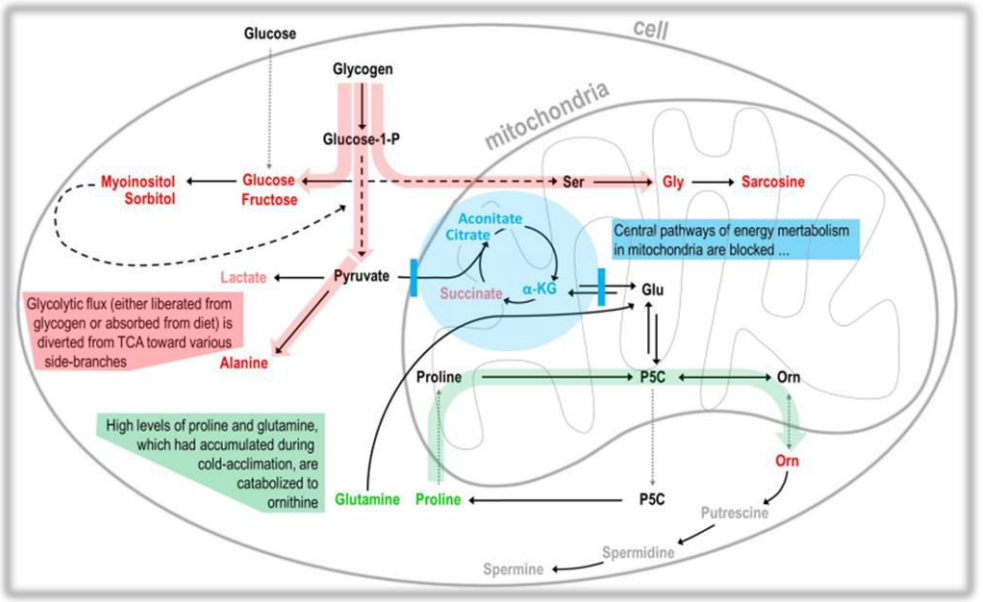

Fig. 1: Schematic summary of major differences found in metabolomic profiles of frozen and cryopreserved larvae of *C. costata* during 3-d-long recovery after cold stress. The Fig. 1 is reproduced from our poster presented at the Annual Meeting of the Society for Experimental Biology, Florence, Italy, 2018.

The analysis of transcriptomic profile suggested that cryopreserved larvae (in comparison to supercooled or frozen larvae) failed to upregulate large number (119) of genes that might be important for averting the delayed mortality during recovery from cold stress. For instance, the whole spectrum of sequences coding key enzymes of the main axis of energy metabolism, centering in mitochondria, was represented, including: carbohydrate digestion (amylase, glucosidase, maltase), glycolysis/ gluconeogenesis (glycogen phosphorylase, fructose-1,6-bisphosphatase, aldolase, triose phosphate isomerase, glyceraldehyde-3-phosphate dehydrogenase, enolase), pentose cycle (phosphogluconate mutase, transketolase),

fermentation (alcohol dehydrogenases, aldehyde dehydrogenase, formaldehyde dehydrogenase, acetyl CoA synthetase), TCA (isocitrate dehydrogenases, succinyl CoA synthetase, succinate dehydrogenase), electron transfer chain (NADH dehydrogenase, ubiquinol-cytochrome c oxidoreductase), and ATP synthesis (ATP synthase). The cryopreserved larvae upregulated only three genes: two genes coding for small heat shock proteins *hsp22* and *hsp26*, and autophagy-specific protein *Atg8a*. The Hsp22 protein is localized in the mitochondrial matrix where it offers protection against oxidative stress (Morrow et al., 2000). The Atg8a is a ubiquitin-like protein localizing to autophagosomes that serve to recycle organelles (such as mitochondria) damaged after various stresses (Zirin and Perrimon, 2010).

Collectively, this study brought several pieces of indirect evidence pointing toward mitochondria as potential targets of freezing stress, which prompted us to have a closer look at responses of *C. costata* mitochondria to cold stress (see chapters 6.3 and 8.3.).

6.2. Recovery from cold stress – *Drosophila melanogaster*

The results were published in the paper:

Štětina T., Poupardin R., Moos M., Šimek P., Šmilauer P., Koštál V. (2019). Larvae of *Drosophila melanogaster* exhibit transcriptional activation of immune response pathways and antimicrobial peptides during recovery from supercooling stress. Insect Biochemistry and Molecular Biology 105, 60-68 (see chapter 7.2.).
Electronic supplementary materials are available at
https://doi.org/10.1016/j.ibmb.2019.01.006

In this paper, we used similar experimental approaches as in our earlier study (Štětina et al., 2018) in order to obtain insight into the processes potentially involved in repair of cold injury in the larvae of vinegar fly (*D. melanogaster*). One response to cold stress has been described relatively well in *D. melanogaster* in the past – the rapid upregulation of HSPs proteins (Goto and Kimura, 1998; Qin et al., 2005; Sinclair et al., 2007; Colinet et al., 2010b). Genetically manipulated flies were used in order to assess the role of HSPs expression in repair of cold injury. Some of these studies suggested significant role for HSPs in recovery after cold stress (Colinet et al., 2010b; Štětina et al., 2015) while the others failed to detect a significant influence of HSPs on survival after cold stress (Nielsen et al., 2005; Udaka et al., 2010). We believe, that such discrepancy is caused mainly by different experimental designs (different genes were considered and, mainly, differently acclimated, different life stages were exposed to different stressful conditions). We previously found, that the upregulation of Hsp70 significantly contributes to survival after only the *severe* and *acute* cold stress but the upregulation of Hsp70

is dispensable (though it occurs) after the *mild* and *chronic* cold stress only when the stress (Štětina et al., 2015). In order to avoid relatively well known HSPs response to cold stress in this study (Štětina et al., 2019), we sampled the insects for transcriptomic analysis 24 h after the stress.

In this study, we assessed recovery from cold stress in the cold-acclimated, quiescent larvae of *D. melanogaster* that were exposed to two physiologically distinct cold stress situations; supercooling and freezing, both at −5 °C. The schematic design of our experiment, together with major outcomes, is depicted in Fig. 2.

The most general outcome of our experiment was that the larvae of *D. melanogaster* showed markedly different metabolic and transcriptomic responses to supercooling and freezing, both at −5 °C. This result was no surprise considering the different severity of both stresses (see chapter 3.1.), which was also reflected in a profound difference in survival: while more than 95% larvae survive until adult stage after supercooling stress, less than 10% survive after the freezing stress. Importantly, most larvae were alive immediately after melting (after freezing stress), the mortality was delayed and occurred after 24 h and later. Therefore, for our analysis we sampled individuals alive at the moment but mostly destined to die.

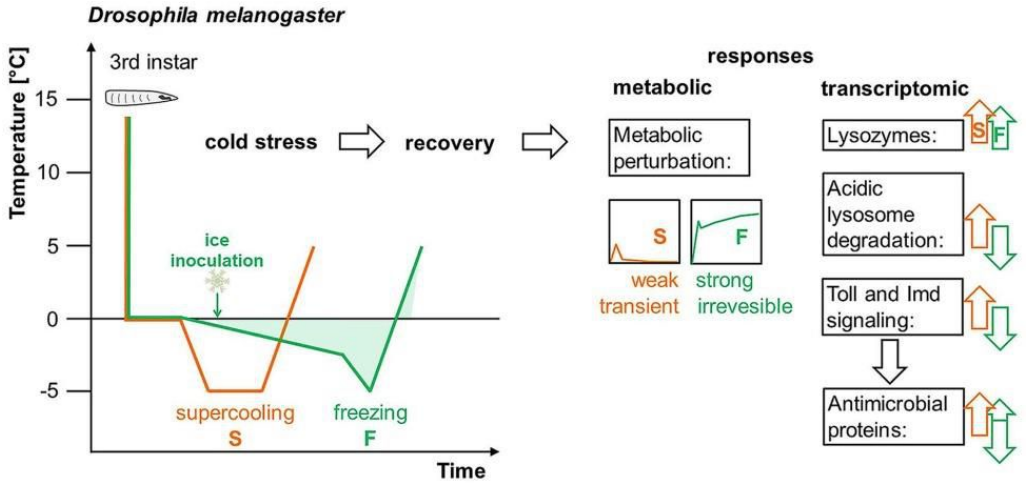

Fig. 2: Schematic design of the experiment on metabolic and transcriptomic responses to cold stress in *D. melanogaster*. The figure is reprint of graphical abstract of the paper by Stetina et al. (2019) published in Insect Biochem. Mol. Biol.

The metabolomic and transcriptomic responses to supercooling stress significantly differed from the profiles of control larvae. This result strongly suggests that the larvae were injured during the supercooling stress but were able to tolerate or repair this injury. Metabolomics analysis revealed that supercooling

was associated with a transient initial perturbation of metabolism. During the first 12 h of recovery after supercooling stress, various metabolites showed rapid fluctuations in concentration that were not seen in controls. Nevertheless, the metabolic profiles of supercooled larvae returned to 'normality', and they were strikingly similar to profiles taken in control larvae screened 24 h or 48 h after cold stress. These results indicate that supercooling was not simply resisted but rather perceived as a sort of stress resulting in disbalance of the metabolic network (Overgaard et al., 2007; Colinet et al., 2012; Williams et al., 2014). Microarray analysis further corroborated the distinction between supercooled and control larvae. The GO term enrichment and KEGG mapping analyses revealed that the immune response pathways and antimicrobial peptides were positively enriched in the response to supercooling. The obvious and complex domination of immune pathways suggests that defense against microbial attack may be an important part of repair processes after the cold stress. It has been reported earlier that cold stress may induce transcriptional immune response in adults of *D. melanogaster* (Zhang et al., 2011; Sinclair et al., 2013; Salehipour-Shirazi et al., 2017). Nevertheless, the analysis of the cold-related immunity in different insects revealed a high level of idiosyncrasy (Ferguson and Sinclair, 2017). It has been shown that low temperatures can both suppress and activate immune responses in ectothermic animals depending on species, overwintering habitat, level of cold stress, type of pathogen, and also depending on which particular metric of immune response is measured (Ferguson et al., 2018).

Metabolomics analysis showed that the perturbation of metabolic networks was deeper in frozen larvae relative to those that were supercooled and, importantly, this perturbation was irreversible in most frozen larvae. The initial phase of recovery (until 6 h after stress) was associated with similar disturbance trends in supercooled and frozen larvae. Afterwards, however, the frozen larvae deviated increasingly from controls and supercooled larvae. The occurrence of delayed mortality was preceded by a peak (at 12 h of recovery) of anaerobic end-products such as alanine, lactate, and succinate. This result suggests that a failure of respiration and aerobic energy production (*i.e.* mitochondrial functions) might be an early event leading finally to systemic failure of whole organism and death. Some elements of transcriptomic responses were shared between supercooled and frozen larvae and many of the shared features can be considered as part of defense responses. Both supercooling and freezing triggered defense mechanisms, but the response to freezing was much less complex or not complete, perhaps interrupted and overwhelmed by death-related processes.

We also realized that the activation of tolerance mechanisms may require energy allotments that may result in impaired fitness (e.g. decreased reproductive output) in cold stress survivors. Similarly, there can be a significant cost linked to unrepaired injury (*i.e.* tolerance cost). The effect of cold stress on fitness has previously been reported in some insects (Coulson and Bale, 1992; Hutchinson and Bale, 1994; Marshall and Sinclair, 2010). Hence, the fitness costs associated with supercooling and freezing stresses in *D. melanogaster* were analyzed in a parallel study (Koštál et al., 2019). The study revealed that the outcome of cold tolerance survival assay profoundly depends on what criterion is taken for survival. If the most often used 'easy' criterion is applied (i.e. survival 24 h after cold stress), then control and supercooled larvae show 100% survival, while frozen larvae show 83% survival. If the delayed mortality is taken into account (*i.e.* survival until adult stage is taken as a criterion), the outcome changes to: 97%, control; 96% supercooled; and 10%, frozen larvae. And if the 'uneasy' but ecologically most relevant criterion is applied (including the fitness of survivor adults, *i.e.* life span and offspring production) the outcomes change dramatically to: 97%, control; 61% supercooled; and 4%, frozen larvae. These result confirmed that repair and/or tolerance of unrepaired injury are real and costly mechanisms/strategies included in the holistic picture of insect cold tolerance and cold hardiness.

6.3. Mitochondria as targets of low temperature-induced injury

The results of this study have been submitted into scientific journal and they are being revised (see chapter 7.3.).

Based on the results of two earlier studies (Štětina et al., 2018; Štětina et al., 2019) we decided to analyze in more detail the responses of mitochondria in *C. costata* to cold stress. Mitochondria play crucial role in function of a living organism including energy supply, metabolic conversions, sensing the changes in cell environment and switching between cell survival or death pathways (Nunnari and Suomalainen, 2012). It has been shown previously that mitochondria are highly plastic and respond physiological and pathological conditions (Vincent et al., 2016). Morphological abnormalities were observed in mitochondria of plants and animals responding to stressful conditions (Virolainen et al., 2002; Abdelwahid et al., 2007; Wang et al., 2016; Jiang et al., 2017). Mitochondrial swelling was observed after freezing stress in plants (Rurek, 2014), mammals (Sherman, 1971; Sherman, 1972) and, in a single case, also in insects – in Malpighian tubules of the freeze-tolerant, winter phenotype larvae of the gall fly, *Eurosta solidaginis* exposed to lethal freezing at -55°C (Zonato et al., 2017). The responses of insect mitochondria to freezing stress were minimally studied so far.

Hence, we decided to compare the sensitivity of mitochondria to freezing stress in a freeze-tolerant model insect, *C. costata*. The relatively freeze-susceptible (non-diapause, warm acclimated) and freeze-tolerant (non-diapause, proline-fed; and diapause, cold-acclimated) phenotypic/acclimation variants of larvae were exposed to cold (-5°C) and freezing stresses (-5°C and -30°C); and the effects of acclimations and cold/freezing stresses on mitochondrial counts, function, and morphology were observed in three larval tissues: fat body, hindgut, and muscle. We found that the acclimation treatments had relatively little influence on mitochondrial function and shape. The mitochondrial counts were slightly (up to 2-fold) reduced upon entry into diapause, but their morphology remained normal and capacity to function (upon transfer to permissive temperature of 23°C) was fully retained even during deep diapause and metabolic suppression at low temperature of 4°C. In contrast, the responses of mitochondria to cold stress profoundly differed in different acclimation treatments. Thus, the mitochondria of freeze-susceptible phenotypic variant exhibited swelling, bursting and loss of respiratory activity when larvae were exposed to lethal freezing at -30°C. In contrast, sublethal freezing or supercooling at -5°C caused only mild or no changes, respectively, in mitochondrial morphology. Similarly, the mitochondria of freeze-tolerant variants of *C. costata* showed only mild or no changes in mitochondrial shape and no loss of respiratory activity upon freezing at -30°C.

Using oxygraph analysis of mitochondrial respiration we found that the enzymatic complexes of the electron transfer chain and ATP synthase remained surprisingly functional even in lethally-injured (dead) larvae of freeze-susceptible phenotype. Similarly, no loss of citrate synthase activity was observed in lethally-injured larvae. These results challenge the paradigm of proteins as primary targets of freezing-induced injury, which is prevented via stabilization of proteins by accumulated cryoprotectants (CPs, see chapter 3.7.) (Tamiya et al., 1985; Carpenter and Crowe, 1988; Privalov, 1990; Franks and Hatley, 1991; Kaushik and Bhat, 2003; Muldrew et al., 2004; Dias et al., 2010; Toxopeus and Sinclair, 2018). In this paper, we suggest alternative hypothetical explanation in which we propose that the phospholipid bilayer might be the primary target of freezing-induced injury. Specifically, we speculate that sudden loss of barrier function (*i.e.* permeability transition and, consequently, swelling) of the inner mitochondrial membrane is caused by unregulated phase transition of phospholipid bilayer upon freeze-dehydration stress. In the freeze-tolerant phenotype of *C. costata*, this transition might be prevented by stabilizing functions of accumulated proline and trehalose (see chapter 3.7.).

7. References

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8. Scientific papers

8.1. Paper I.

Štětina T., Hůla P., Moos M., Šimek P., Šmilauer P., Košťál V (2018). Recovery from supercooling, freezing, and cryopreservation stress in larvae of the drosophilid fly, *Chymomyza costata*. Scientific Reports 8, 4414.

SCIENTIFIC REPORTS

OPEN

Recovery from supercooling, freezing, and cryopreservation stress in larvae of the drosophilid fly, *Chymomyza costata*

Received: 19 January 2018
Accepted: 27 February 2018
Published online: 13 March 2018

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Physiological adjustments accompanying insect cold acclimation prior to cold stress have been relatively well explored. In contrast, recovery from cold stress received much less attention. Here we report on recovery of drosophilid fly larvae (*Chymomyza costata)* from three different levels of cold stress: supercooling to −10 °C, freezing at −30 °C, and cryopreservation at −196 °C. Analysis of larval CO_2 production suggested that recovery from all three cold stresses requires access to additional energy reserves to support cold-injury repair processes. Metabolomic profiling (targeting 41 metabolites using mass spectrometry) and custom microarray analysis (targeting 1,124 candidate mRNA sequences) indicated that additional energy was needed to: clear by-products of anaerobic metabolism, deal with oxidative stress, re-fold partially denatured proteins, and remove damaged proteins, complexes and/or organelles. Metabolomic and transcriptomic recovery profiles were closely similar in supercooled and frozen larvae, most of which successfully repaired the cold injury and metamorphosed into adults. In contrast, the majority of cryopreseved larvae failed to proceed in ontogenesis, showed specific metabolic perturbations suggesting impaired mitochondrial function, and failed to up-regulate a set of 116 specific genes potentially linked to repair of cold injury.

Insects evolved complex and efficient strategies for survival at body temperatures below the equilibrium melting point of their body liquids[1,2]. The insect cold tolerance literature has primarily focused on physiological mechanisms that accompany seasonal or rapid cold hardening and help to prevent occurrence of cold injury[3]. Thus, cold hardening has been associated with global changes in gene transcription, protein expression, and metabolome composition[4]; transition from active life to developmental arrest called diapause[5]; accumulation of low-molecular weight cryoprotectants[6]; synthesis of proteins which regulate the process of ice formation[7]; compositional remodeling of cell membranes[8]; and rearrangement of cytoskeleton structure[9,10]. Adapted and properly acclimated cold hardy insects are often *a priori* considered to be resistant to the occurence of cold injury. Nevertheless, their cold hardiness might also be based, at least partly, on their abilty to tolerate or even actively repair the injury that potentially incurred during the cold stress.

Participation of active repair processes in recovery from cold stress is supported by observations of cold-induced upregulation of the activity of cellular protective systems preventing apoptosis[11], oxidative damage[12,13], and loss of proteins' native conformation[14]. The activation of heat shock protein (HSP) production is the most typical immediate physiological response to environmental stress observed in almost all organisms[15] including insects exposed to cold[16–23]. HSPs are ubiquitous molecular chaperones that can prevent the irreversible aggregation of cold-denaturing proteins in an ATP-independent manner[24], or assist in protein re-folding and protein cellular degradation in an ATP-dependent manner[25]. At the insect survival level, the participation of active repair processes in recovery from cold stress is supported by observation of 'delayed mortality' – a mortality which does not occur immediately upon cold stress but instead later during ontogenesis[26–28]. Though delayed mortality is considered as critically important for ecologically meaningful interpretation of survival assays[29,30], it is often neglected in experimental practice because of costs linked with maintaining the insect culture for long periods of time following treatment[29]. The delayed mortality might be, in theory, regarded as an inability to repair any

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Parameter	Units	Sex	Control (C)	Cold stress		
				Supercooling (S)	Freezing (F)	Cryopreservation (LN)
Survival analysis						
Survival*, larva, 12 h	%	n.a.	100 (272)	99.2 (200)	100 (300)	84.5 (200)
Survival, puparium	%	n.a.	97.3	99.0	95.7	48
Time to pupariation	*days*	n.a.	*18.7 ± 0.4 (14.0–25.2)*	n.a.	n.a.	n.a.
Survival, adult	%	n.a.	93.6	95.0	89.3	39.5
Time to adult emergence	*days*	n.a.	*30.9 ± 0.03 (27.1–38.0)*	n.a.	n.a.	n.a.

Table 1. Survival of *Chymomyza costata* larvae after different cold stresses. *Numbers of larvae used for survival analysis (*n*) are shown in parentheses. Data on developmental timing (in *italics*) are shown as mean ± S.D.; in addition, the range is shown in parentheses for developmental timing. n.a., not analyzed.

vital injury that occurred, or was triggered, during previous exposure to cold. Additionally, the cost of cold-injury repair processes may be manifested as a reduction in fitness for survivors (i.e. offspring production), which has also previously been demonstrated in some studies[31–33]. We argue that additional studies on recovery from cold stress are greatly needed in order to understand the whole process of insect cold tolerance.

Here, we report on recovery from cold stress in the larvae of drosophilid fly, *Chymomyza costata*. We used diapausing larvae acclimated to a relatively low temperature of 4 °C because such larvae are physiologically 'uniform' and exhibit the highest level of cold hardiness[34]. We exposed the larvae to one of three different levels of cold stress: supercooling to −10 °C (S), freezing at −30 °C (F), or cryopreservation in liquid nitrogen at −196 °C (LN), and observed changes in their metabolomic and transcriptomic profiles for three days of recovery from cold stress, at 18 °C. Transferring the control, non-stressed larvae (C) to 18 °C was used to subtract the responses linked to resumption of locomotion, feeding, metabolic activity, and continuation in ontogenesis toward pupation. In addition, we estimated the rate of resumption of larval activity upon transfer to 18 °C by analyzing how they balance cold-induced hyperkalemia (via measurement of hemolymph potassium concentrations) and how they increase their metabolic rate (via monitoring of CO_2 production). We expected that increasing the dose of cold stress (S < F < LN) will increase the proportion of delayed mortality in *C. costata* larvae, as different stresses may cause quantitatively and/or qualitatively different injuries. Specifically, we aimed to obtain insight into the nature of cold injuries and their repair based on metabolomic and transcriptomic profiling. We hypothesized that: (I) The metabolomic and transcriptomic profiles will be similar in C and S larvae, as there is almost no delayed mortality in these variants (i.e. supercooling does not seem to cause any injury and requires no repair). (II) The metabolomic and transcriptomic profiles will be different between S and F larvae, as the conditions of S and F treatments widely differ. The F treatment includes growth of extracellular ice crystals, freeze-induced cellular dehydration, and shrinkage associated with a whole array of deleterious consequences[35], while the S larvae experience 'only' a decrease of temperature; the cell volume, water activity, and associated parameters remain relatively stable. (III) The metabolomic and transcriptomic profiles will be similar in F and LN larvae, as they experience similar (high) magnitude of freeze-induced dehydration. The potential differences between F and LN larvae will help to identify structures and processes vulnerable to injury caused by rapid changes of temperature during cryopreservation.

Results and Discussion

Larvae are not instantaneously killed by cold stress but may die later in ontogenesis. Survival of larvae, checked 12 h after the transfer to 18 °C, was relatively high in all experimental variants (control and three different cold-stress treatments), ranging between 84.5% and 100% (Table 1). Larvae pupariated on average 18.7 days after the transfer to 18 °C, and another 12.2 days were required for pupal metamorphosis and adult emergence (Table 1). Some individuals perished later during the development, many days or even weeks after the end of cold stress. This delayed mortality was low or practically absent in the C, S, and F experimental variants (where 93.6, 95.0 and 89.3% of larvae survived to adult stage, respectively). In contrast, delayed mortality was relatively high in cryopreserved larvae (LN), where only 39.5% of larvae were able to metamorphose into adults (Table 1).

Recovery from cold stress requires energy. We observed that almost all cold-stressed larvae were able to restore their locomotor activity relatively rapidly upon transfer to 18 °C. In fact, many larvae (irrespective of treatment) actively crawled in a fully coordinated way already within the period of handling after the end of cold stress, prior to the start of physiological measurements. Restoration of locomotor behavior requires coordinated neuromuscular activity, which in turn depends on normal (i.e. uneven) distribution of ions across biological membranes. It is a well-known fact that cold exposure dissipates transmembrane electrochemical potentials in non-adapted and/or non-acclimated animals[36,37]. Adapted and cold-acclimated ectotherms, however, maintain transmembrane electrochemical potentials when chilled or even supercooled for long periods of time, which is regarded as one of the crucial physiological mechanisms supporting their cold tolerance[38–40]. In freeze-tolerant insects, however, the transmembrane electrochemical potentials dissipate during freezing and are restored rapidly upon melting[41]. Hemolymph hyperkalemia (increasing $[K^+]$) is thus a typical sign of disturbed ionic regulation both during insect freezing[41] and lethal supercooling[40,42–44].

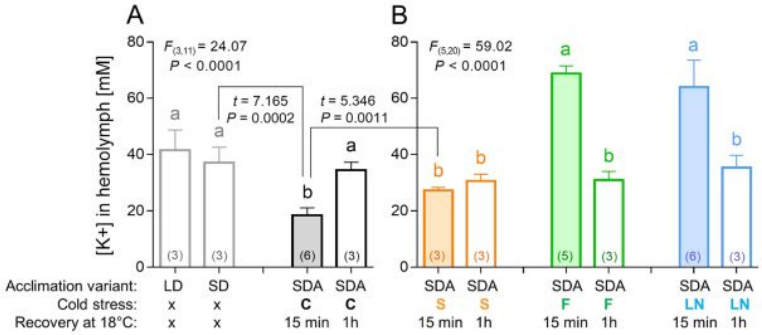


Figure 1. Hemolymph potassium concentration in control and cold-stressed *Chymomyza costata* larvae. Each column shows the mean (and S.D.) of $[K^+]$ analyzed using an ion-selective K^+ microelectrode in a sample of hemolymph collected from a pool of 10–20 larvae (the number of pools, i.e. biological replicates are shown in parentheses). The larvae were variously acclimated: LD, fully active and developing, warm-acclimated; SD, diapausing, warm-acclimated; and SDA, diapausing, cold-acclimated. (**A**) The control larvae, C, were not subjected to cold stress but were transferred from 4 °C directly to 18 °C and the $[K^+]$ was measured 15 min or 1 h after the transfer. (**B**) The cold-stressed larvae were: S, supercooled to −10 °C; F, frozen at −30 °C; or LN, cryopreserved in liquid nitrogen. After the cold stress, the larvae were transferred to 18 °C and the $[K^+]$ was measured 15 min or 1 h after the transfer. Differences between treatments were analyzed using ANOVA followed by Bonferroni's test (columns flanked by different letters are statistically different). The control treatments (**A**) and cold-stressed treatments (**B**) were analyzed separately and *F* statistics and *P* values are presented. In addition, t-tests were used to compare two specific treatments (connected by zig-zag lines) and *t* statistics and *P* values are shown. See text for more details.

We found $[K^+]$ of 41.5 mM in hemolymph of active, directly developing larvae of *C. costata* (acclimation variant LD; for explanation of acclimation variants, see Fig. S1). A similar value (37.1 mM) was seen in warm-acclimated, diapausing larvae (acclimation variant SD). The cold-acclimated diapausing larvae (acclimation variant SDA) exhibited significantly lower $[K^+]$ of 18.5 mM but it rapidly increased (within 1 h) to 34.4 mM upon transfer to 18 °C (Fig. 1A). In theory, the relatively low $[K^+]$ in SDA larvae might have an adaptive meaning as it would help to counteract hyperkalemia during exposures to cold extremes[45]. Supercooled larvae (S) displayed a $[K^+]$ corresponding to untreated controls. In contrast, frozen (F) and cryopreserved (LN) larvae showed significantly higher $[K^+]$ when measured 15 min after the end of cold stress: 68.7 mM and 64.0 mM, respectively. These results demonstrate that larval freezing is associated with dissipation of transmembrane electrochemical potentials, similarly as it was shown previously in frozen larvae of the fly, *Xylophagus cincta*[41]. At 1 h of recovery, the larvae of all treatments showed similar $[K^+]$: 30.6 mM (S); 31.0 mM (F); and 35.3 mM (LN), which is close to a value observed in 18 °C-exposed controls (Fig. 1B). These results show that frozen larvae were able to fully restore dissipated electrochemical potentials within 1 h after the cold stress. Such exercise undoubtedly required energy supply for the activity of primary ion pumping ATPases, which establish ionic homeostasis.

Next, we measured CO_2 production as a direct proxy of metabolic rate and energy turnover during recovery from cold stress (Fig. 2, see Dataset S1 for more details). In all experimental variants, handling the larvae prior to the start of measurement took approximately 30 min during which no data were taken. During the first 30-min interval of measurement (30–60 min after cold stress), the values of CO_2 production (µl of CO_2 produced per mg FM per interval) significantly differed among the variants: 1.73 ± 0.42 (C); 1.23 ± 0.35 (S); 0.70 ± 0.45 (F), 0.75 ± 0.84 (LN) (ANOVA $F_{(3,20)} = 4.683$; $P = 0.0123$), and Bonferroni's multiple comparisons post hoc test found significant differences between C vs. F and also C vs. LN variants. These results document that larvae which underwent freezing event (F and LN) were recovering slightly slower than control larvae (C). The values of CO_2 production were similar in all variants within the second interval of measurement (60–90 min after cold stress) ranging between 1.99 and 2.29 µl CO_2 (ANOVA $F_{(3,20)} = 0.7073$; $P = 0.5589$). These results confirm that larval recovery from cold stress is relatively fast in all treatments. An apparent overshoot of CO_2 production was detectable during first hours of recovery in all treatments. The overshoot was most probably associated with high locomotion activity stimulated by handling and transfer to light and 18 °C (we observed that larvae vigorously crawled and climbed the walls of rearing tubes during the first hours after the transfer).

Figure 2 suggests that there were no profound differences among four experimental variants in the overall pattern of CO_2 production over 3 days. Nevertheless, after summing up the total production of CO_2 over the whole 3 day period, all three treatments showed higher values than control. A difference of 48 µl.mg^{-1} FM of CO_2, as found between S and C larvae, was statistically significant (239.2 vs. 191.2 µl.mg^{-1} FM of CO_2, respectively). A typical SDA larva has a FM of approximately 2 mg and carries approximately 40–50 µg of glycogen and 250–300 µg of total lipids[34]. In theory, one larva would need to oxidize 80 µg of carbohydrate (glycogen) or 140 µg of lipidic energy substrate (calculated using the coefficients by Schmidt-Nielsen[46]) to produce 2 × 48 = 96 µl of CO_2 (per 2 mg of FM). This calculation suggests that considerable part of energy turnover (approximately 20%)

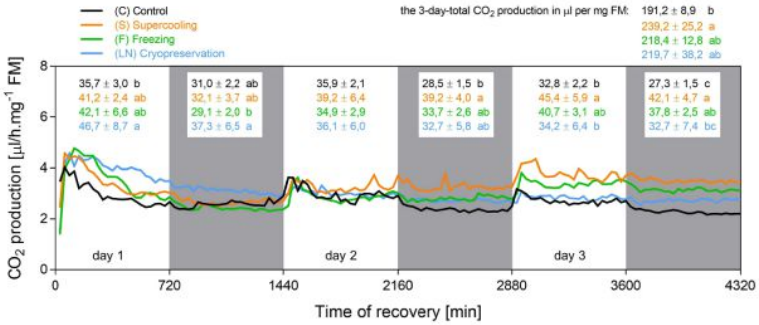


Figure 2. CO_2 production in control and cold-stressed *Chymomyza costata* larvae. Each line represents a mean record of CO_2 production analyzed in six groups of ten larvae. The record was taken in 30 min intervals during three days (4,320 min) of recovery at 18 °C from different cold stresses (S, F, LN, see Fig. 1 and text for more details). The control larvae (C) were not subjected to cold stress but were transferred from 4 °C directly to 18 °C. White and grey areas represent alteration of light and dark conditions (12 h L, 12 h D). The numbers show mean ± S.D. CO_2 production is expressed in µl per mg fresh mass (FM) for each experimental variant (color coded) during 12 h intervals and also during the whole three day period (means flanked by different letters are statistically different according to ANOVA followed by Bonferroni's test). See Dataset S1 for more details.

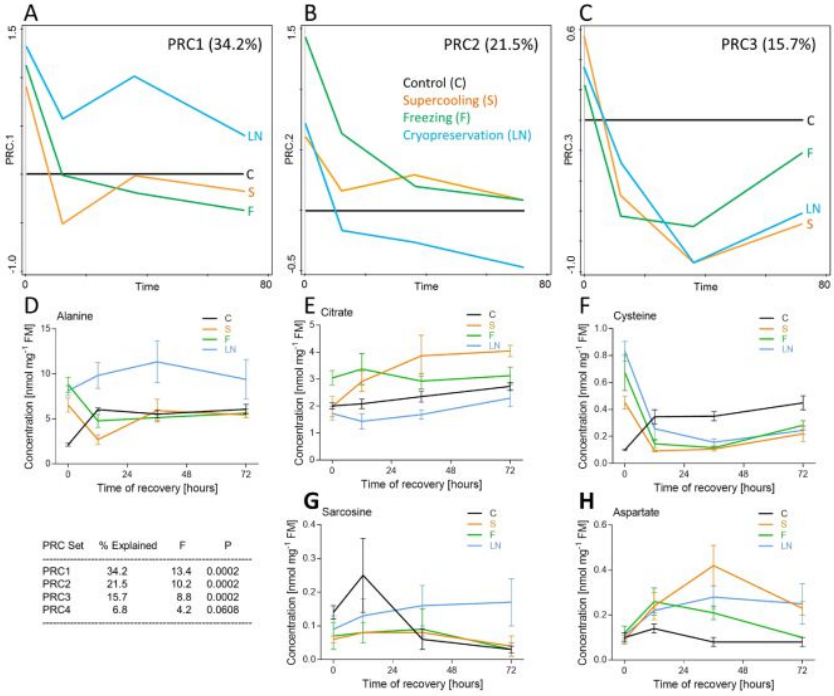
in a larva recovering from cold stress is devoted to the repair processes. This is agreement with other studies[31–33] that indirectly demonstrate the existence of a cost of cold-injury repair processes in the form of impaired fitness of survivors (reduced reproductive capacity). It seems as counter-intuitive that the cost of repair processes was highest in S larvae while it was lower in F and LN larvae that experienced 'harsher' treatments. We can offer a speculative explanation that an unknown proportion of living (during analysis) but doomed larvae in F and (especially) LN treatments (see Table 1) might cause an underestimation of the true cost of repair after the freezing and cryopreservation stress. Collectively, our data and the literature suggest that insect recovery from cold stress requires access to energy, which is used to re-establish the homeostasis at warm conditions and to repair cold-induced injuries.

In addition, we analyzed the patterns of CO_2 production in order to detect presence of daily rhythmicity and any potential differences in this parameter. Visual inspection of the patterns on Fig. 2 suggests that larvae exhibit only very weak daily rhythmicity in CO_2 production at best. A small difference between day and night CO_2 production rates is visually detectable in all experimental variants. The presence of a peak in CO_2 production stimulated by light ON is also clearly detectable in all variants. Results of statistical analysis of daily rhythmicity are presented in the supplementary information (Dataset S1). Based on these results, we can conclude that the cold stress did not interfere with neuromuscular responsiveness of *C. costata* to a 'morning' light stimulus.

Metabolite profiling revealed themes common to all treatments but also separated the LN vs. S and F treatments. Results of targeted metabolomics are summarized in Dataset S2. As demonstrated in Fig. S1 (Dataset S2, Excel sheet: Results), the sum concentrations of amino-compounds, sugars and polyols, and organic acids (intermediates of glycolysis and TCA cycle) remained relatively stable and did not differ substantially among the experimental variants. Four of five principal metabolites (those which together represent > 87% of the total concentration of all 41 analyzed metabolites), namely proline, glutamine, trehalose, and asparagine, showed similar temporal profiles in all four variants (Fig. S2, Dataset S2, Excel sheet: Results). The fifth principal metabolite, alanine, will be discussed later.

Statistical analysis (PRC) was used to detect leading patterns in temporal metabolomic profiles and, mainly, to identify where and how these patterns differ among treatments. The proportion of explained variation for a single particular metabolite exceeded our arbitrary threshold value Cfit > 0.5 in only two cases (alanine and fructose in PRC set 1). The magnitudes of responses were relatively low, i.e. exceeding the arbitrary threshold value of Resp > ± 2.0 in only three cases (alanine in PRC set 1, and cysteine and aspartate in PRC set 3), which led us to consider a less stringent threshold value of Resp > ± 1.5. Applying this less-stringent threshold, 18 metabolites (37.5% of 41 targeted metabolites) exhibited temporal patterns of concentration changes during recovery deviating prominently between treated (S, F, and LN) vs. control (C) larvae (Dataset S2, Excel sheet: PRC analysis). The PRC analysis thus revealed that the recovery from cold stress (irrespective of the treatment) differs from a mere resumption of larval activities upon transfer from low to high temperature. The PRC model had three PRC sets showing statistically significant proportion of variation explained by the main effect of treatment plus its interaction with time (Fig. 3A–C). The fourth PRC set, though also marginally statistically significant, is not presented in Fig. 3 as the proportion of explained variation (6.8%) was relatively low. For each PRC set, we present examples of the best-predicted metabolites according to a combination of Resp and Cfit parameters (Fig. 3D–H).

The PRC set 1 (Fig. 3A) shows a difference in temporal patterns between LN vs. S and F treatments. This difference was best represented by alanine (Fig. 3D), fructose and several other metabolites (for details, see



PRC Set	% Explained	F	P
PRC1	34.2	13.4	0.0002
PRC2	21.5	10.2	0.0002
PRC3	15.7	8.8	0.0002
PRC4	6.8	4.2	0.0608

Figure 3. Metabolite profiles of control and cold-stressed *Chymomyza costata* larvae. The targeted metabolomics of 41 different metabolites was conducted using a combination of mass spectrometry-based analytical methods. (**A–C**) The principal response curve (PRC) model found three principal PRC sets showing statistically significant proportion of variation explained by the main effect of treatment plus its interaction with time. In control larvae (C), the temporal pattern of metabolic changes during their 3-day recovery after transfer to 18 °C is levelled to 0 and the temporal patterns of three cold-stressed treatments (S, F, LN) are normalized to the control. (**D–H**) Examples of real concentration changes during recovery from cold stress in selected best-predicted metabolites are shown for each PRC set (observe columns below PRCs 1, 2, 3). See text and Dataset S2 for more details.

Dataset S2, Excel sheet: PRC analysis). At time 0, their concentrations were higher than control in all three treatments. Later during recovery, however, the concentrations either dropped below or close to control values (S and F) or remained relatively high or even increased (LN). The PRC set 2 (Fig. 3B) detected, again, a difference between LN vs. S and F treatments, which was represented best by citrate (Fig. 3E), ketoglutarate, and some other metabolites on one side (pattern PRC2a) vs. sarcosine (Fig. 3G) on the other side (pattern PRC2b). In the pattern PRC2a, initially high levels of a metabolite decreased during recovery but either remained above control (S and F) or dropped below control (LN). The pattern PRC2b was opposite: initially low levels increased during recovery but either remained close to control (S and F) or increased significantly above control (LN). The PRC set 3 (Fig. 3C) separated the temporal profiles according to quantitative differences in concentrations, while the directions of responses (higher/lower than control) were similar in all treatments. The pattern PRC3a associated metabolites such as cysteine (Fig. 3F), succinate, and lactate, showing very high concentrations at time 0 followed by a rapid drop below the control levels during recovery. In contrast, the pattern PRC3b associated metabolites such as aspartate (Fig. 3H), glutamate, and glycine, showing higher concentrations in treatments than in control during recovery.

While interpreting the results of metabolomics, we noticed commonalities and differences among three different cold stress treatments. The strong commonality suggests that all three cold stresses exhibited partially overlapping effects on larval physiology (caused similar sort of cold injury) that were not observed in control larvae. The differences unexpectedly draw the main division line between S and F vs. LN rather than between supercooled (S) vs. frozen (F and LN) larvae.

Commonalities. The larvae of all cold treatments sampled at time 0 showed clear symptoms of a past anaerobic episode in their metabolism, such as high lactate, succinate, and alanine concentrations in comparison to control[47,48]. In addition, all cold-treated larvae showed high levels of cysteine at time 0, which might

indicate a disturbance of redox homeostasis and oxidative stress[49,50]. During the next hours of recovery, anaerobic by-products were rapidly cleared (with a single exception: alanine in LN), and cysteine concentrations decreased below the control levels, indicating return to aerobic homeostasis. As another commonality, concentrations of aspartate and glutamate transiently increased during recovery in cold-treated variants in comparison to control. The aspartate and glutamate may serve as sinks for amino groups in transamination reactions during catabolism of all amino acids[51]. Therefore, transiently increasing levels of glutamate and aspartate might be associated with increasing rates of protein degradation which, in turn, may reflect higher rates of removal of cold-injured proteins and cold-induced chaperones[14,25]. As chaperoning requires ATP, this result indirectly supports our data on increased energy requirement during recovery from cold stress.

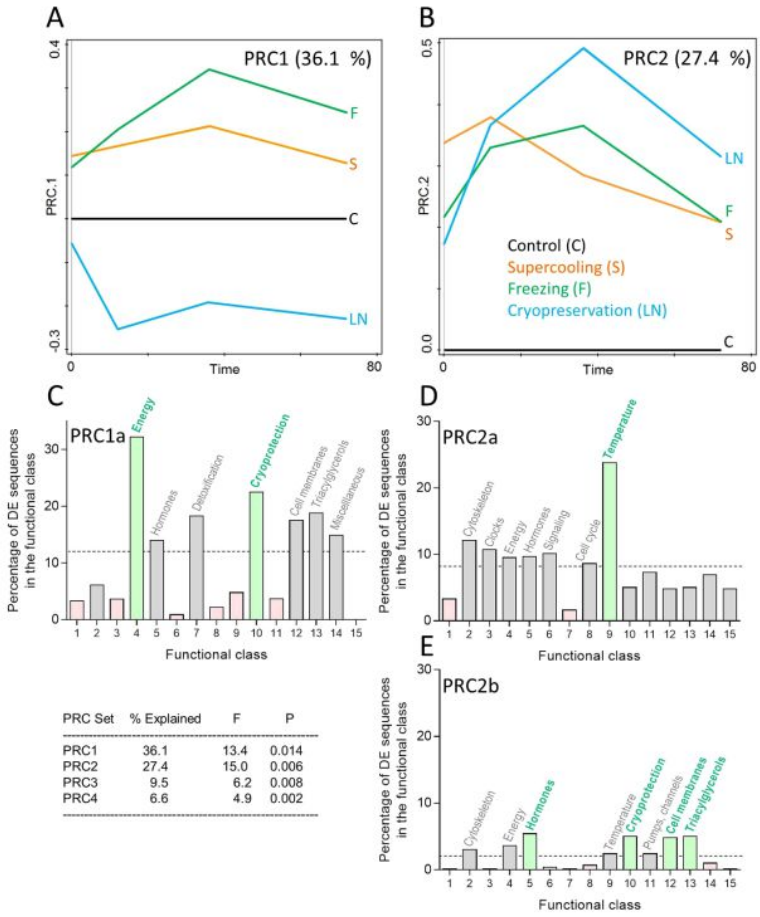
Differences. The metabolic perturbations observed specifically in the LN treatment seem to point toward impaired mitochondrial function during unsuccessful recovery/delayed mortality following cryopreservation: (i) The accumulations of glucose and several derivates of the glycolytic pathway side-branches, such as fructose, myo-inositol, sorbitol, and glycine, suggest that the glycolytic flux (liberated from glycogen and/or absorbed from diet) is partially diverted from TCA and production of energy toward accumulation of alanine[52]. (ii) The catabolism of lactate and cysteine, that were accumulated during the previous anaerobic episode, might contribute to production of excess pyruvate, which again might be canalized preferentially to alanine rather than to TCA. (iii) The level of sarcosine (a derivative of glycine) has been reported to increase in cancer cells, known to divert their glycolytic flux from TCA to anaerobic end-products[53]. (iv) The relatively low levels of citrate, aconitate, and ketoglutarate might result from partial blockade of pyruvate entry to, or early steps of, the TCA cycle in the mitochondrial matrix [see (i) – (iii)][48]. (v) The accumulation of ornithine (the synthesis of which is also located inside mitochondria) might indicate re-routing of mitochondrial catabolic pathways for excess amino acids (proline, glutamine, glutamate) from TCA (partially blocked) toward ornithine[54]. At the same time, the polyamines (putrescine, spermine, and spermidine), to which ornithine can be further transformed upon decarboxylation in the cytosol, were undetectable or found in traces in all treatments. (vi) The last segment of the TCA cycle, catabolism of succinate[48], that accumulated during a previous anaerobic episode, seems to operate normally in LN larvae.

Gene transcript profiling revealed general transcriptional upregulation during recovery but again separated the LN vs. S and F treatments. Results of targeted transcriptomics are summarised in Dataset S2. According to PRC analysis, 223 differentially expressed (DE) sequences (19.8% of the 1,124 candidate sequences represented on the custom microarray) exhibited temporal patterns of expression during recovery deviating significantly between cold-treated (S, F, and LN) vs. control (C) larvae. Therefore, recovery from cold stress was clearly identified as an exercise differing in many aspects from a mere resumption of metabolic, behavioral, and developmental activities upon transfer of (control) larvae from low to high temperature. For filtering the DE sequences, we applied the criteria: Resp $> \pm 2.0$, and Cfit > 0.5 (Dataset S2, Excel sheet: Sequences and PRC analysis). The PRC model had two PRC sets showing statistically significant proportions of variation explained by the main effect of treatment plus its interaction with time (Fig. 4A,B). Two other PRC sets, PRC3 and PRC4, though also statistically significant, are not presented in Fig. 4 as the proportions of explained variation (9.5% and 4.4%, respectively) were relatively low.

The PRC set 1 (Fig. 4A) separated the LN treatment from the other two cold treatments (S and F) according to temporal patterns of 119 DE sequences. Most (116) of PRC1 DE sequences were upregulated during recovery in S and F treatments, but down-regulated (or not different from control) in the LN treatment (pattern PRC1a). Several examples of the predicted DE sequences are shown in Fig. S3 (Dataset S2, Excel sheet: PRC1 DE sequences). The Seq. 81398, *ornithine decarboxylase 1* (*odc1*) was the best-predicted PRC1 DE sequence (Cfit = 0.81; and Resp = 3.05), and the temporal patterns of its expression were validated by RT-qPCR method (Fig. S5, Dataset S2, Excel sheet: RT-qPCR validation). Only three PRC1 DE sequences showed an opposite pattern (PRC1b, not shown in figure): upregulated during recovery from LN treatment, while not different from control in S and F treatments (pattern PRC1b). The Seq. 60138, *heat shock protein 22* (*hsp 22*) was the best-predicted (Cfit = 0.42; and Resp = −3.34) of the three sequences (Fig. S3, Dataset S3, Excel sheet: PRC1 DE sequences; validation: Fig. S15, Dataset S3, Excel sheet: qRT-PCR validation). Looking at 116 PRC1a DE sequences according to functional categories, two classes emerged as most often represented (enriched classes): Energy and Cryoprotection (Fig. 4C).

The PRC set 2 (Fig. 4B) separated the treatments according to quantitative differences in sequence expression while the directions of responses (higher/lower than control) were similar in all treatments. The PRC2a pattern associated 94 sequences that were up-regulated in all three treatments during recovery in comparison to control. This pattern was dominated by a functional class Temperature (Fig. 4D) and the Seq. 53852, *centromere identifier* was the best-predicted example with Cfit = 0.86, and Resp = 5.05 (Fig. S4, Dataset 2, Excel sheet: PRC2 DE sequences; validation: Fig. S5, Dataset S3, Excel sheet: qRT-PCR validation). The PRC2b pattern associated 24 sequences that were down-regulated in all treatments during recovery in comparison to control. This pattern was driven by several functional classes (Fig. 4E) and the Seq. 3875, *histidine decarboxylase* was the best-predicted example with Cfit = 0.70, and Resp = −2.78 (Fig. S4, Dataset 2, Excel sheet: PRC2 DE sequences; validation: Fig. S5, Dataset S3, Excel sheet: qRT-PCR validation). Like in the metabolomics dataset, the transcriptomics dataset suggested that there are strong commonalities and some differences in the temporal patterns of gene expression during recovery from three different cold stresses.

Commonalities. Transcriptional upregulation dominated over downregulation during recovery. Both PRC sets 1 and 2 were driven mainly by DE sequences showing upregulation-type response in comparison to control



PRC Set	% Explained	F	P
PRC1	36.1	13.4	0.014
PRC2	27.4	15.0	0.006
PRC3	9.5	6.2	0.008
PRC4	6.6	4.9	0.002

Figure 4. Gene transcript profiles of control and cold-stressed *Chymomyza costata* larvae. The transcriptomics analysis was conducted using custom C. costata microarrays containing probes for 1,124 candidate gene sequences. (**A**,**B**) The principal response curve (PRC) model found two principal PRC sets showing relatively high and statistically significant proportion of variation explained by the main effect of treatment plus its interaction with time. In control larvae, C, the temporal pattern of transcriptional changes during their 3-day recovery after transfer to 18 °C is levelled to 0 and the temporal patterns of three cold-stressed treatments (S, F, LN) are normalized to the control. (**C**–**E**) The differentially expressed (DE) sequences for each PRC set (1, 2) are clusterred in functional classes and percentages of DE genes within each functional class (enrichment) is scored. See Dataset S3 for calculation of the percentages of DE sequences and for more details.

(Fig. 4A,B and Figs S3 and S4). The upregulated DE sequences most often fall into three functional classes: Energy, Temperature, and Cryoprotection (Fig. 4C,D) which we interpret in a following way: As transcriptional upregulation generally consumes energy, the results support existence of energetic cost of repair processes. The repair processes might be linked with clearance of by-products of anaerobic metabolism, return of redox homeostasis, dealing with oxidative stress [all suggested by metabolomic profiling (Fig. 3) and also by literature[13,55,56]], and re-folding or removal of proteins that were partially denatured during cold stress [again reflected in metabolomic profiling (Fig. 3) and literature[22]]. The catabolism of putative cryoprotectants, such as proline, glutamine and trehalose, might differ (in terms of preferred pathways and/or rates of catabolism) between control and cold-treated larvae. The cryoprotectants were accumulated in large quantities during the long-term cold acclimation prior to cold stress[34], and might serve as alternative energy substrates during subsequent ontogenesis[6,57]. Our metabolomics profiling, however, did not suggest any rapid clearance of cryoprotectants during the first three days of recovery from cold stress (Fig. S2). The elevated Cryoprotection DE transcripts, and the clearance of cryoprotectants, may thus come into effect only later during ontogenesis, as the larvae require 2–3 weeks for pupariation (Table 1).

Differences. We interpret the results of PRC set 1a as suggesting that LN larvae, in contrast to S and F larvae, failed to upregulate a number of sequences that might be important for successful repair of cold injury and averting the delayed mortality during recovery from cold stress. In the most enriched functional class, Energy (32.2% DE sequences, Fig. 4C), the whole spectrum of sequences coding for key enzymes of the main axis of intermediary and energy metabolism is represented, including: carbohydrate digestion (amylase, glucosidase, maltase), glycolysis/gluconeogenesis (glycogen phosphorylase, fructose-1,6-bisphosphatase, aldolase, triose phosphate isomerase, glyceraldehyde-3-phosphate dehydrogenase, enolase), pentose cycle (phosphogluconate mutase, transketolase), fermentation (alcohol dehydrogenases, aldehyde dehydrogenase, formaldehyde dehydrogenase, acetyl CoA synthetase), TCA (isocitrate dehydrogenases, succinyl CoA synthetase, succinate dehydrogenase), electron transfer chain (NADH dehydrogenase, ubiquinol-cytochrome c oxidoreductase), and ATP synthesis (ATP synthase). In addition, a number of sequences in the class Triacylglycerols (18.8% DE sequences, the third most enriched class in PRC1a) may also fall within the broader category of 'intermediary metabolism' as they include sequences coding for synthesis and degradation of lipids centered around metabolism of acyl-CoA. The second most enriched class in PRC1a, Cryoprotection (22.5% DE sequences), also reflects well the metabolomics results showing differential regulation of ornithine concentrations. The best-predicted sequence of the class Cryoprotection is *ornithine decarboxylase 1*, and number of other DE sequences code for elements of ornithine metabolism (ornithine aminotransferase, pyridoxal-5-phosphate synthetase, glutamine synthetases, glutamate transporter). The fourth most enriched class in PRC1a, Detoxification (18.3% DE sequences) includes a number of sequences coding for enzymes responsible for coping with oxidative stress and redox signalling (catalase, superoxide dismutase, glutathione synthetase, glutathione S transferase, thioredoxine peroxidases, peroxiredoxins, methionine sulfoxide reductase). This class is also reflected in the metabolomics dataset in the differentially-regulated levels of cysteine, glycine, and glutamate (three components of glutathione).

There were only three sequences upregulated specifically in LN-treated larvae and not in the other two treatments (pattern PRC1b). Two of them, *hsp22* and *hsp26*, code for small heat shock proteins and *Atg8a* codes for an autophagy-specific protein. The small heat shock proteins stabilize early unfolding intermediates of aggregation-prone proteins, arising as a result of diverse stress conditions including cold shock[24,58]. The Hsp22 protein is localized in the mitochondrial matrix and its high level of expression in aging flies suggested a role in protection against oxidative stress[59]. Relatively high expression of different HSP-coding sequences was characteristically associated with recovery from all three cold stresses (see pattern PRC2a, enriched functional class Temperature, Fig. 4D). Thus, high expression of *hsp22* and *hsp26* specifically in the LN treatment may only confirm that protein denaturation was most severe in this particular treatment. The *Atg8a* codes for ubiquitin-like protein localizing to autophagosomes[60]. Autophagosomes serve to recycle large protein complexes and damaged organelles during insect metamorphosis, periods of starvation, or in response to injuries caused by oxidative stress, pathogenic infection, misfolded proteins, and hypoxia[60,61]. Transcriptional upregulation of *Atg8a*, and other autophagy-related genes, is characteristically observed just prior to cell death (induced developmentally or in response to stress) in various cell types in *D. melanogaster*[62,63]. High expression of *Atg8a* in the LN treatment is thus another marker of severe injury caused by cryopreservation stress.

Conclusions

Contrary to our expectation, the metabolomic and transcriptomic profiles of recovery from cold stress differed between control and supercooled larvae (hypothesis I), were similar in supercooled and frozen larvae (hypothesis II), and differed between frozen and cryopreserved larvae (hypothesis III). PRC analysis draws two main division lines between: (i) control vs. all cold-stressed variants, and (ii) supercoooled and frozen (with high adult survivorship) vs. cryopreserved (exhibiting high proportion of delayed mortality) variants.

Despite the fact that diapausing, cold-acclimated *C. costata* larvae belong among the most cold-hardy animals on Earth[34], our results suggest that they are injured by all three cold treatments. While most (> 89.3%) of supercooled and frozen larvae were able to repair the injuries and successfully metamorphosed into adults, cryopreserved larvae exhibited relatively high (60.5%) levels of delayed mortality that occurred days or weeks after the end of cold stress. Repair processes required access to additional energy (larval CO_2 production increased during recovery) exceeding the standard requirement by approximately 20%. Metabolomic profiling and custom microarray analyses provided hints on repair processes. In comparison to control larvae, all three cold stresses caused increased concentrations of anaerobic by-products (alanine, lactate, and succinate), metabolic markers of oxidative stress (cysteine), and amino-acid sinks for amino groups (glutamate, aspartate). In addition, 94 sequences were up-regulated while 24 sequences were down-regulated during recovery from all stresses. Among the upregulated sequences, the gene functional category 'response to temperature stress', including various heat shock proteins, was the most enriched. The metabolic perturbations observed specifically in cryopreserved larvae point toward impaired mitochondrial function and these larvae also failed to upregulate a set of 116 sequences that were up-regulated in supecooled and frozen larvae. These sequences covered functional categories of intermediary and energy metabolism, metabolism linked to potential cryoprotectants (proline, glutamine, trehalose), dealing with oxidative stress, and re-folding or removal of partially denatured proteins. In contrast, three sequences were specifically up-regulated in cryopreserved larvae: two small heat shock proteins *hsp22* and *hsp26*, and an autophagy-related gene *Atg8a*. Collectively, our results suggest that repair and disposal of damaged proteins is an important feature of recovery from cold stress in *C. costata* larvae.

Methods

Insects, diapause, cold-acclimation. Adult *Chymomyza costata* (Diptera: Drosophilidae) flies were originally collected in the wild in 1983, in Sapporo (43.1°N, 141.4°E), Hokkaido, Japan. Since then, the insect culture has been maintained in the laboratory on an artificial diet as described earlier[64] in MIR154 incubators (Sanyo Electric, Osaka, Japan) under conditions promoting direct development, i.e. constant temperature of 18 °C and

a long-day photoperiod (LD, Light:Dark phase, L16 h:D8 h). Diapause was induced in experimental cohorts of larvae by rearing them at a constant temperature of 18 °C and a short-day photoperiod (SD, L12 h:D12 h). Under these conditions, all individuals respond reliably to photoperiodic signal and enter into diapause as fully grown 3rd instar larvae[65–68]. Next, diapausing larvae were cold acclimated by transferring them to 11 °C and constant darkness at 6 weeks of age and, two weeks later, transferring them to 4 °C for another 4 weeks. This gradual, 6-week cold acclimation (SDA) dramatically enhances freeze tolerance such that frozen larvae survive when cryopreserved in liquid nitrogen (LN_2)[34].

Exposure to cold stress, survival. Twelve-week-old, cold-acclimated, diapausing larvae (SDA) were either directly sampled for analyses (control, C) or were exposed to one of three different cold stresses in a programmable Ministat 240 cooling circulator (Huber, Offenburg, Germany): supercooling (S) to −10 °C; freezing (F) to −30 °C; or cryopreservation (LN) in LN_2 (for schematic depiction of the experimental design, see Fig. S1). Larvae destined for cold exposure were separated from the larval diet by washing in ice-cold water and groups of approximately 20 larvae were placed in between two layers of cellulose (75 mg) that were moistened with 300 µl of distilled water. The moist cellulose 'ball' with larvae inside was inserted into a plastic tube (diameter, 1 cm; length, 5 cm), and the tube placed in the cooling circulator at the start of temperature program. The temperature inside the cellulose ball was continuously monitored in control tubes (containing no larvae) with K-type thermocouples connected to a PicoLog TC-08 datalogger (Pico Technology, St. Neots, United Kingdom). To ensure *supercooling* conditions for the S treatment, a 50% glycerol solution was used to moisten the cellulose ball instead of distilled water. Glycerol prevents the occurrence of spontaenous freezing of water inside the ball and we verified in preliminary experiments that glycerol has no effect on larval survival (neither positive nor negative). To ensure *freezing* conditions in the F tyreatment, a small ice crystal was added to the surface of the moist cellulose (at the start of the temperature program), which results in immediate freezing of water inside the ball and stimulates ice penetration and inoculative freezing of larval body fluids. To assess *cryopreservation*, the larvae gradually frozen to −30 °C were plunged in LN_2 for one hour and the returned back to −30 °C. The temperature programs were described in detail previously[34,35], and are schematically presented on Figure S1. At the end of program, the unpacked cellulose balls were transferred on fresh standard diet in a tube maintained at constant 18 °C. Alive/dead larvae were scored after 12 h recovery. All living larvae were maintained at 18 °C for a subsequent 6 weeks and succesful pupariation and emergence of adult flies were scored as ultimate criterions of survival. In addition, we measured the fresh mass (FM) and dry mass (DM, after three days of drying at 60 °C) in 40 survivor adults (20 males, 20 females) from the control and cold treatments. Exact numbers of larvae used for each specific experiment are shown in Results.

Although we sampled only the apparently living specimens (those moving spontaneously and crawling in a coordinated manner), we cannot exclude the possibility that an unknown proportion of sampled specimens were in fact doomed and, therefore, displaying metabolomic and transcriptomic changes leading to death rather than to repair of cold injury and successful recovery. This drawback is inherently present in all studies where only a fraction of individuals survive the treatment.

Potassium ions concentration in hemolymph and CO2 production. In order to estimate the rate of recovery from cold stress in control and cold-exposed larvae, we analyzed the concentration of potassium ions $[K^+]$ in the hemolymph, and the production of CO_2. Hemolymph $[K^+]$ was measured using an MI-442 K^+ Ion Microelectrode in combination with a reference electrode MI-402 (both from Microelectrodes Inc., Bedford, NH, USA). A sample of hemolymph was collected from a pool of 10–20 larvae (to reach ca. 3 µl in total) into calibrated micro-capillary tube (Broomall, PA, USA). Exactly 2.5 µl of hemolymph was then diluted 3 times with 5 µl of deionized water in order to obtain sufficient volume for microelectrodes (7.5 µl). Three to six biological replicates (pools of 10–20 larvae) were measured as soon as possible after the end of cold stress treatment (first sampling) and one hour after the end of cold stress treatment (second sampling). The manipulation of 10–20 larvae (unwrapping the cellulose ball, tearing, and collecting the hemolymph into the capillary) took approximately 15 min at room temperature. Voltage was measured using pH/mV Hand-Held Meter pH 330 (WTW, Weilheim, Germany) and converted to $[K^+]$ using a semilog line regression calibration curve. The calibration samples (1 mM, 10 mM, 100 mM KCl solution) were measured just prior to measuring the hemolymph samples on every occasion. The one-way ANOVA was used to analyze whether there is any influence of the treatment on $[K^+]$ and the post-hoc Bonferroni's multiple comparisons tests were applied to find the differences among particular treatments. Data were initially tested for normality (Kolmogorov-Smirno test) and homoscedascity (Bartlett's test) before subjecting them to ANOVA. Unpaired two-tailed *t*-tests were used to assess the differences in $[K^+]$ between two specific treatments. The *F* tests were applied first to verify that variances of the two menas do not significantly differ. These statistical calculations were performed using Prism6 (GraphPad software, San Diego, CA, USA).

The production of CO_2 was measured using the respirometry analysis system MFC-2/TR-SS3/MUX (Sable Systems International, Las Vegas, NV, USA) equipped with LI 7000 CO_2/H_2O analyzer (LI-COR Biosciences, Bad Homburg, Germany). Six groups of 10 larvae of known FM (for each experimental variant) were placed in six glass tubes (volume 22 ml) on 1 g of larval diet that was sterilized by application of PenStrep (Sigma-Aldrich, Saint Luis, MO, USA) in concentrations 100 U peniciline and 0.1 mg streptomycine per 10 g diet. Two another tubes served as blank controls (an empty glass tube, and a glass tube containing PenStrep-treated diet only). All tubes were placed inside the MIR154 incubator set to a constant temperature of 18 °C and a short-day photoperiod (SD) where Zeitgeber time 0 was set to the moment when the cold exposure finished. The manipulations following completion of the cold exposure program (unwrapping the cellulose ball, counting larvae, weighing FM, and transferring larvae to glass tubes) took approximately 30 min, and the analysis of CO_2 production started immediately after. Larvae lived inside tubes for 72 h and their CO_2 production was measured in 30 min intervals. During

each interval, the system was 'closed' for 1,575 sec and then flushed for 225 sec using 75 ml of CO_2-devoid air from a tank (Linde Gas, Praha, Czech Republic). The CO_2 was removed using CO_2-absorbent soda lime (Elemental Microanalysis, Okehampton, UK). We used the ExpeData software tool (v.1.2.02, Sable Systems International) to process the CO_2 data. After 72 h, surviving larvae were weighed a second time and the two FM records were used to calculate the CO_2 in $\mu l/h.mg^{-1}$ FM assuming a linear increase in FM during the three days (survival was typically 100%; if not, the FM of dead larvae was subtracted from calculations for second half of the three day period). The differences in CO_2 production among treatments were assessed using ANOVA followed by Bonferroni's test as described above (Prism6). In addition, we searched for daily rhythmic pattern in CO_2 production data by visual inspection of the data and also using two statistical methods, a shareware program CircVawe v.1.4 (Roelof A. Hut, University of Groningen, Netherlands, http://www.rug.nl/fwn/onderzoek/programmas/biologie/chronobiologie/downloads/index), and a Lomb-Scargle P_s periodogram[69,70]. The raw data for CO_2 production and results of statistical analysis are presented in Dataset S1.

Targeted metabolomics. Pools of five larvae (in four biological replicates) were sampled at four time points of recovery from cold stress: 0, 12, 36, and 72 h (see Fig. S1). The first sample (time 0) was taken at low temperature (4 °C in control; 5 °C, in cold treatments). The larvae were weighed for FM, plunged into LN_2, and stored at −80 °C until analysis. The pools of larvae were homogenized in 400 µl of a methanol:acetonitrile:water mixture (volumetric ratio, 2:2:1) containing internal standards (*p*-fluoro-DL-phenylalanine, methyl α-D-glucopyranoside; both at a final concentration of 200 $nmol.ml^{-1}$; both from Sigma-Aldrich, Saint Luis, MI, USA). The TissueLyser LT (Qiagen, Hilden, Germany) was set to 50 Hz for 5 min (with a rotor pre-chilled to −20 °C). Homogenization was repeated twice and two supernatants stemming from centrifugation at 20,000 g/5 min/4 °C were combined. The extracts were subjected to targeted analysis of major metabolites using a combination of mass spectrometry-based analytical methods described previously[56].

Low-molecular-weight sugars and polyols were determined after *o*-methyloxime trimethylsilyl derivatization using a gas chromatograph (GC) with flame ionization detector GC-FID-2014 equipped with AOC-20i autosampler (both from Shimadzu Corporation, Kyoto, Japan). Profiling of acidic metabolites was done after treatment with ethyl chloroformate under pyridine catalysis and simultaneous extraction in chloroform[71]. The analyses were conducted using Trace 1300 GC combined with single quadrupole mass spectrometry (ISQ) (both from Thermo Fisher Scientific, San Jose, CA, USA) and a liquid chromatograph Dionex Ultimate 3000 coupled with high resolution mass spectrometer Q Exactive Plus (all from Thermo Fisher Scientific). All metabolites were identified against relevant standards and subjected to quantitative analysis using a standard calibration curve method. All standards were purchased from Sigma-Aldrich. The analytical methods were validated by simultaneously running blanks (no larvae in the sample), standard biological quality-control samples (the periodic analysis of a standardized larval sample – the pool of all samples), and quality-control mixtures of amino acids (AAS18, Sigma Aldrich).

The results of targeted metabolomics (as summarized in Dataset S2, Excel sheet: Raw data) were subjected to statistical analysis based on constrained linear ordination methods using Canoco software (v. 5.04)[72]. Concentrations of metabolites were first log-transformed (using a $ln(100*y+1)$ formula) and then centered and standardized. We used a specialized multivariate method called principal response curves (PRC) which focuses on time-dependent treatment effects on multivariate response data[73]. Each principal component (corresponding to first and higher axes of the underlying partial RDA) is plotted against time, yielding one PRC curve for each experimental variant. In addition, the temporal pattern of metabolomic changes in the control variant (C) was set as a reference value (0) and the temporal patterns of the treatments (S, F, LN) represent differences from the control.

Custom microarray analysis. Transcriptomic profiling was based on 1,124 candidate sequences arbitrarily selected from published Illumina RNAseq database that contains 21,327 putative mRNA transcripts of *C. costata* (ArrayExpress accession E-MTAB-3620). The sequences were annotated and manually classified into 15 functional categories based on GO terms, InterPro, FlyBase, KEGG and other descriptions[74]. The selected genes broadly cover major structures and processes known, or suggested, to be linked to insect diapause and cold tolerance. The methods for custom microarray production were described previously[68]. We added 79 new sequences, putatively involved in DNA repair and processing of unfolded proteins, to the second generation of custom microarray (Cos2 microarray). The complete list of Cos2 microarray sequences is presented in supplementary material (Dataset S3, Excel sheet: Sequences and PRC analysis).

Pools of five larvae were sampled at four time points of recovery from cold stress: 0, 12, 36, and 72 h (see Fig. S1). There were two levels of replication: technical triplicates of each spot on the microarray (1, 2, 3), and biological replication of triplicate samples for each treatment (separate pools of larvae: a, b, c). The first sample (time 0) was taken at low temperature (4 °C in control; 5 °C, in cold treatments). Sampled larvae were stored in RiboZol RNA Extraction Reagent (Amresco, Solon, OH, USA) at −80 °C prior to processing. All details of sample processing were the same as published earlier[68]. Briefly: Total RNA was isolated using RiboZol and treated with DNase I (Ambion, Life Technologies, Foster City, CA, USA). For the first strand cDNA synthesis, we used the $Oligo(dT)_{23}$ anchored primer (Sigma-Aldrich) and Superscript III (Invitrogen, Carlsbad, CA, USA). The second DNA strand was synthesized using DNA polymerase I and treated with RNaseH (both Invitrogen). Next, double strand DNA was purified using Wizard SV Gel and PCR Clean-up System (Promega, Madison, WI, USA). The double strand DNA was labelled with Cyanine-5-dCTP dye (Cy5, PerkinElmer, Waltham, MA, USA) using Klenow fragment polymerase and the BioPrime DNA Labelling System (Invitrogen). After removing the unincorporated dye (Illustra AutoSeq G-50 Dye Terminator Removal Kit, GE Healthcare, Little Chalfont, UK) and adding a blocking agent (sonicated salmon sperm DNA, Invitrogen), the labelled double strand DNA was used for hybridization onto microarrays. An automatic Hybridization Station HS4800 Pro (Tecan, Mannedorf, Switzerland) was

used to perform all the hybridization steps (washing the station, pre-hybridization, hybridization, rinsing and drying). All microarrays were scanned immediately upon completion of the hybridization procedure using the ScanArray G_x Microarray Scanner (PerkinElmer) at a resolution of 5 µm and the fluorescence was quantified using ScanArray Express v. 4.0.0.0004 software (Perkin Elmer). Fluorescence values were normalized between arrays using Quantile normalization[75]. All log2-normalized fluorescence values are summarized in Dataset S3 (Excel sheet: Raw data). Next, mean log2-normalized fluorescence values were calculated by averaging the spot technical triplicates and the resulting dataset was used for principal response curves (PRC) analysis (Canoco 5) similarly as described for metabolites.

We selected four sequences for technical validation of microarray analysis results by quantitative real time PCR (qPCR) using a CFX96 PCR cycler (BioRad, Philadelphia, PA, USA). The relative mRNA transcript abundance of selected target sequences was measured in the aliquots of the same total RNA as subjected previously to microarray analysis. The total RNA treated with DNase I (Ambion) was used for the first strand cDNA synthesis primed with Oligo$(dT)_{23}$ anchored primer (Sigma-Aldrich) using Superscript III (Invitrogen). The cDNA products (20 µL) were diluted 25 times with sterile water. PCR reactions (total volume of 20 µL) contained 5 µL of diluted cDNA template, LA Hot Start Plain Master Mix (TopBio, Vestec, Czech Republic), and were primed with a pair of gene-specific oligonucleotide primers (Table S1, Dataset S3, Excel sheet: qRT-PCR validation), each supplied in a final concentration of 400 nM. Cycling parameters were 3 min at 95 °C followed by 40 cycles of 94 °C for 15 s, 60 °C for 30 s and 72 °C for 30 s. Analysis of melt curves verified that only one product was amplified in each reaction. In addition, we checked the size of the PCR products for each gene by electrophoresis on 2% agarose gel in selected samples. Emission of a fluorescent signal resulting from SYBR Green binding to double-stranded DNA PCR products was detected with increasing PCR cycle number. Threshold cycle (C_T) for each sample was automatically calculated using the algorithm built in the CFX96 PCR light cycler software. We used two genes coding for Ribosomal proteins, *RpL19* and *RpL32* as endogenous reference standards for relative quantification of the target transcript levels[68]. Each sample was run in duplicate (two technical replicates) of which the mean was taken for calculation. Relative ratios of the target mRNA levels (C_T) to geometric mean of the levels (C_T) of two reference gene mRNAs were calculated as ddC_T[76].

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Acknowledgements

We thank Jaroslava Korbelová, Irena Vacková, Anna Heydová, Iva Opekarová, and Helena Zahradníčková (all from Biology Centre CAS) for assistance with insect rearing, sample preparation and analyses. Konrad Schöttner (Concordia University, Montreal) performed statistical analysis of daily rhythmicity in larval CO_2 production. Lauren Des Marteaux (Biology Centre CAS) commented on an early version of the paper. This work was supported by the Czech Science Foundation (grant 16–06374 S to V.K.).

Author Contributions

V.K. designed the research approach, directed the project, adjusted and validated experimental procedures, analyzed data, assembled all figures, and wrote the manuscript. T.Š. measured potassium concentrations, CO_2 production, and performed the microarray analysis. P.H. recorded larval survival and performed the RT-qPCR validation. M.M. and P. Šimek developed, assessed, and conducted the metabolomic analysis. P. Šmilauer performed the PRC statistical analyses of metabolomic and transcriptomic data.

Additional Information

Supplementary information accompanies this paper at https://doi.org/10.1038/s41598-018-22757-0.

Competing Interests: The authors declare no competing interests.

Publisher's note: Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Recovery from supercooling, freezing, and cryopreservation stress in larvae of the drosophilid fly, *Chymomyza costata*.

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Supplementary Information

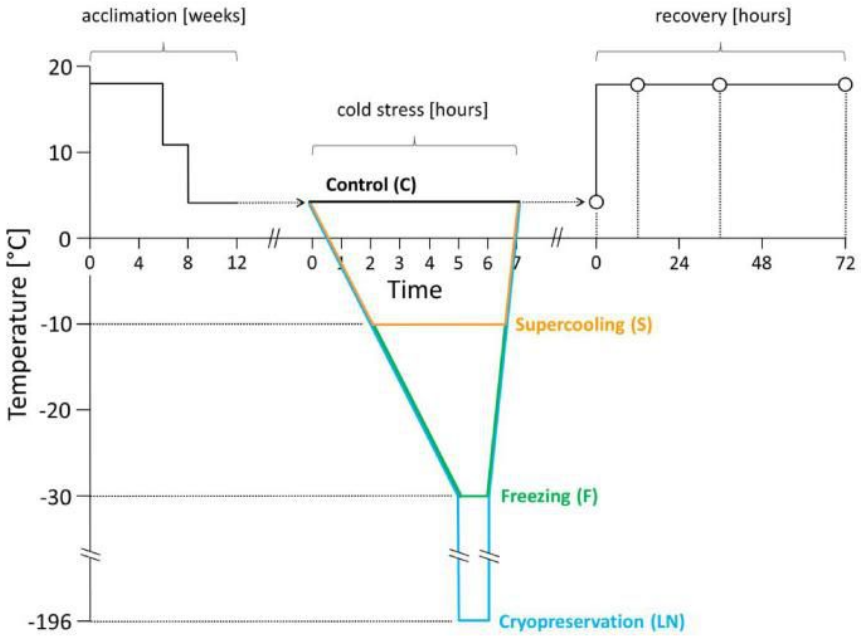

Figure S1. Schematic depiction of experimental design used to analyze the metabolomic and transcriptomic responses in control and cold-stressed larvae of *Chymomyza costata*. Most of the experimental larvae were diapausing, cold-acclimated (SDA) meaning that they were reared under short days and 18°C for 6 weeks and, subsequently, gradually cold acclimated at 11°C for 2 weeks followed by 4 weeks at 4°C. Next, the SDA larvae were either directly transferred to 18°C (control, C) or were cold-stressed. Three levels of cold stress were tested: (S), supercooling to -10°C; (F), freezing at -30°C; or (LN), or cryopreservation in liquid nitrogen. After the cold stress, the larvae were transferred to 18°C for recovery. During the recovery from cold stress, the concentrations of hemolymph potassium, CO_2 production, and profiles the metabolome and transcriptome were assessed (at times 0, 12, 36 and 72 h of recovery).

Two other acclimation variants (LD and SD) were specifically used as controls for analysis of $[K^+]$. The LD larvae came from continuous culture maintained under conditions promoting direct development, *i.e.* constant temperature of 18°C and a long-day photoperiod. The SD larvae are 6-week old larvae reared under short days and 18°C, *i.e.* early diapausing, warm acclimated.

8.2 Paper II.

Štětina T., Poupardin R., Moos M., Šimek P., Šmilauer P., Koštál V. (2019). Larvae of *Drosophila melanogaster* exhibit transcriptional activation of immune response pathways and antimicrobial peptides during recovery from supercooling stress. Insect Biochemistry and Molecular Biology 105, 60-68.

Insect Biochemistry and Molecular Biology 105 (2019) 60–68

Contents lists available at ScienceDirect

Insect Biochemistry and Molecular Biology

journal homepage: www.elsevier.com/locate/ibmb

Larvae of *Drosophila melanogaster* exhibit transcriptional activation of immune response pathways and antimicrobial peptides during recovery from supercooling stress

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ARTICLE INFO

Keywords:
Vinegar fly
Drosophila
Cold hardiness
Metabolome
Gene expression
Immune response

ABSTRACT

The biochemical and molecular mechanisms underlying insect cold acclimation prior to cold stress are relatively well explored, but the mechanisms linked to recovery and repair after cold stress have received much less attention. Here we focus on recovery from cold stress in the larvae of the vinegar fly (*Drosophila melanogaster*) that were exposed to two physiologically distinct cold stress situations: supercooling (S, survival > 95%) and freezing (F, survival < 10%), both at −5 °C. We analysed the metabolic and transcriptomic responses to cold stress via GC-MS/LC-MS and whole-genome microarrays, respectively. Both stresses (S and F) caused metabolic perturbations which were transient in supercooled larvae but deeper and irreversible in frozen larvae. Differential gene expression analysis revealed a clear disparity in responses to supercooling and freezing (less than 10% of DE genes overlapped between S and F larvae). Using GO term enrichment analysis and KEGG pathway mapping, we identified the stimulation of immune response pathways as a strong candidate mechanism for coping with supercooling. Supercooling caused complex transcriptional activation of innate immunity potential: from Lysozyme-mediated degradation of bacterial cell walls, recognition of pathogen signals, through phagocytosis and lysosomal degradation, Toll and Imd signaling, to upregulation of genes coding for different antimicrobial peptides. The transcriptomic response to freezing was instead dominated by degradation of macromolecules and death-related processes such as autophagy and apoptosis. Of the 45 upregulated DE genes overlapping in responses to supercooling and freezing, 26 were broadly ascribable to defense and repair functions.

1. Introduction

Insects are excellent animal models for studies on cold hardiness–the capacity to survive subzero body temperatures (Denlinger and Lee, 2010; Lee and Denlinger. 1991). Two broad categories of cold hardiness are commonly distinguished: insects may (i) supercool, i.e. maintain the body water in a liquid phase at subzero temperatures (the most common strategy) or (ii) survive freezing, i.e. survive formation of ice crystals inside the body (Salt, 1961; Sinclair et al., 2003; Zachariassen, 1985). This simple dichotomous classification, although long-debated in the field, will be sufficient for the purpose of the present study. In the scientific literature, considerable attention has been paid to analysis of physiological mechanisms underlying insect cold hardiness. Most studies have focused on physiological changes that are triggered by either seasonal acclimation and acclimatization (lab- and field-studies, respectively) or rapid cold hardening, i.e. *prior* to cold stress (see reviews; Lee, 2010; Overgaard and MacMillan, 2017; Teets and Denlinger, 2013; Toxopeus and Sinclair, 2018; Zachariassen, 1985). Such changes were mostly interpreted as preventing the occurrence of cold injury (i.e. increasing *resistance* to cold injury). For instance, accumulation of antifreeze proteins prevents freezing occurrence (Duman, 2015), accumulated small cryoprotective molecules stabilizes proteins and other

Abbreviations: DE, differential expression; F, freezing; FTR, fluctuating thermal regime; GO, gene ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; PRC, principal response curve; RDA, redundancy analysis; S, supercooling

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https://doi.org/10.1016/j.ibmb.2019.01.006
Received 26 November 2018; Received in revised form 14 January 2019; Accepted 14 January 2019
Available online 17 January 2019

macromolecular structures (Timasheff, 2002), membrane restructuring can protect against unregulated phase transitions (Koštál, 2010), and altered activity of ion pumps and channels can prevent dissipation of membrane potentials upon cold exposure (Bayley et al., 2018). In addition to resistance mechanisms, cold hardiness also depends on an insect's ability to *tolerate* and/or repair the incurred injury (Koštál et al., 2019). For example, a rapid upregulation of various heat shock proteins (HSPs) is often observed after cold stress, and it is believed that this response helps in the re-folding or removal of proteins that were partially denatured during the cold stress (Rinehart et al., 2007; Štětina et al., 2018; Koštál and Tollarová-Borovanská, 2009; Zhao and Jones, 2012). The activation of tolerance mechanisms often requires energy allotments that may result in impaired fitness (e.g. decreased reproductive output) in cold stress survivors (Coulson and Bale, 1992). Hence, considering both resistance and tolerance mechanisms, the survival *and* the fitness of survivors dictate the final outcome of cold stress (Koštál et al., 2019). We argue that the contribution of tolerance mechanisms to insect cold hardiness is relatively understudied and we hope to ignite more interest in the analysis of physiological events *after* the cold stress, during the recovery period.

Here, we analyse the cold stress response of the vinegar fly (*Drosophila melanogaster*), and use untargeted metabolomics and transcriptomics approaches to obtain insight into the processes potentially involved in repair of cold injury. Specifically, we use the larval stage of *D. melanogaster*, for which detailed knowledge about cold hardiness was obtained in our earlier studies (Koštál et al., 2011, 2012; 2016a, 2016b; Rozsypal et al., 2018; Štětina et al., 2015). As a species of tropical origin, the vinegar fly is relatively susceptible to cold stress (Strachan et al., 2010). Nevertheless, it was shown that various simple laboratory acclimations, such as rapid cold hardening (a brief pre-treatment at sublethal low temperature prior to cold stress), long-term developmental cold acclimation (rearing the insects at a relatively low but developmentally-permissible temperature), and induction of quiescence (by exposing the larvae to temperatures just below the lower developmental threshold), dramatically increase the resistance of *D. melanogaster* to cold stress (Czajka and Lee, 1990; Jensen et al., 2007; Koštál et al., 2016a, 2016b; Rajamohan and Sinclair, 2008; Rako and Hoffmann, 2006). To explain this acclimation-linked increase in cold hardiness, several resistance mechanisms have been suggested for *D. melanogaster* (although most await direct functional validation): the accumulation of cryoprotective metabolites (such as proline and trehalose) (Koštál et al., 2011); modification of membrane phospholipid composition (Koštál et al., 2011; Overgaard et al., 2005, 2008); adjustments to the ionoregulatory system (MacMillan et al., 2015). Among potential tolerance mechanisms, the upregulation of HSP expression after cold stress received relatively high attention. In addition to several descriptive studies (Colinet et al., 2010a; Goto et al., 1998; Sinclair et al., 2007; Qin et al., 2005), functional validations were performed using genetically manipulated flies with altered expression of HSPs. However, the outcomes of validation studies were not straightforward; some suggested a weak role for HSPs in recovery after cold stress (Colinet et al., 2010b, 2010c; Štětina et al., 2015), while the others failed to detect a significant influence of HSPs on survival after cold stress (Nielsen et al., 2005; Udaka et al., 2013). The transcriptional activation of HSPs is a typical short-term response to cold stress. In the present study, by sampling insects 24 h after the cold stress we avoided the time window during which the transcriptional activation of HSPs is observed (Štětina et al., 2015). In this way, we hoped to detect other, long-term tolerance mechanisms that might be involved in repair of cold stress injury.

In the present study, we focus on recovery from cold stress in the cold-acclimated, quiescent larvae of *D. melanogaster* that were exposed to two physiologically distinct cold stress situations; supercooling (S) and freezing (F), both at −5 °C. Control larvae (C, not exposed to cold stress) served as a reference. The untargeted approach was used to describe the metabolic (via GC-MS/LC-MS analyses) and transcriptomic (via whole-genome microarrays) responses to cold stress linked to either successful recovery (> 95% survival to adult stage in supercooled larvae) or unsuccessful recovery (> 90% delayed mortality in frozen larvae). We asked two major questions: (i) How do the larvae survive after supercooling? Do they *resist* (avoid) the cold injuries (?), in which case we would not expect to see many differences between supercooled larvae and controls. Alternatively, do they *tolerate* (repair) cold injury (?), in which case we expected to obtain hints of what was damaged and/or what the repair mechanisms are. (ii) What characterizes the larvae exposed to freezing stress? Do they exhibit metabolic and transcriptomic responses that overlap with those observed in supercooled larvae (?), which would reveal general mechanisms that are induced irrespectively of the nature of the cold stress. Or do they show freezing-specific responses (?), which would mainly indicate the changes linked to severe freezing injury and mortality, typically occurring with a delay (> 1 day after stress).

2. Materials and methods

2.1. Insects and experimental design

All experiments were conducted in parallel with another study (Koštál et al., 2019) which focused on survival and fitness parameters of adults subjected to cold stress as larvae. The culture of vinegar flies, *Drosophila* (*Sophophora*) *melanogaster* of Oregon R strain (Lindsley and Grell, 1968) was maintained at a constant 18 °C and LD cycle of 12 h L/12 h D on agar-sugar-yeast-cornmeal diet in MIR 154 incubators (Panasonic Healthcare, Gunma, Japan). For experiments, the larvae were acclimated at a constant 15 °C and LD cycle until the age of 11 days (pre-wandering stage) and then moved to acclimation conditions under constant darkness and a fluctuating thermal regime (FTR) of 6 °C for 20 h/11 °C for 4 h, for three days. Under FTR conditions, larvae o *D. melanogaster* enter quiescence (i.e. developmental arrest induced directly by low temperature) and their cold hardiness increases (Koštál et al., 2016b). Note that control larvae (C) also experienced the FTR treatment in order to 'subtract' the effects of FTR on metabolic networks and gene expression patterns.

For a schematic depiction of the sampling plan and experimental design, see Fig. 1. The quiescent larvae were washed out of the diet and the first sample of larvae for metabolomics analysis (a pool of 10 larvae in each of 4 biological replicates) was taken prior to cold stress (at time

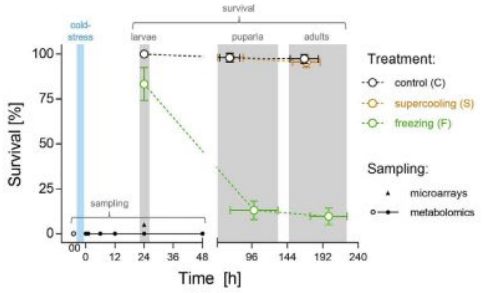


Fig. 1. Sampling plan and design of the experiment on metabolic and transcriptomic responses to cold stress in larvae of *Drosophila melanogaster* (S, supercooling; F, freezing; C, unstressed control). The first sample for metabolomics was taken prior to cold stress (time 00) and other samples were taken during recovery after cold stress (at 0, 1, 6, 12, 24, and 48 h). A single sample for microarray analysis was taken at 24 h of recovery after cold stress. The data on survival after the supercooling and freezing stresses were taken from a parallel study (Koštál et al., 2019). Each point represents a mean ± SD percent survivors and also a mean ± SD time of developmental transition (n = C, 300, C; S, 280, S; F, 900 larvae).

00). All remaining larvae were exposed to cold stress or served as controls. Control larvae were directly transferred into a new rearing vial with fresh larval diet and placed at constant 15 °C (i.e. no cold stress). Cold-stressed larvae were exposed to −5 °C as described elsewhere (Koštál et al., 2019):

- supercooling (S): 0 °C for 1 h, followed by ramping down to −5 °C over 30 min, maintaining at a constant −5 °C for 60 min, then ramping up to +5 °C over 40 min; or
- freezing (F): 0 °C for 1 h, followed by inoculative freezing stimulated by a small ice crystal, and ramping down to −2.5 °C over 180 min, then ramping down to −5 °C over 30 min, and finally ramping up to +5 °C over 40 min.

Larval body fluids remained liquid throughout the S treatment, and were partially frozen in the F treatment (Koštál et al., 2012; Rozsypal et al., 2018). In the parallel study (Koštál et al., 2019), analogously treated larvae were maintained to score their survival to puparial and adult stages, and to follow fitness parameters in survivor adults. The survival data are reproduced in Fig. 1 for clarity.

The cold-stressed larvae were moved to new rearing tubes with fresh diet and placed at constant 15 °C and LD cycle. Samples for metabolomics were taken at 0 h (immediately after cold stress), 1 h, 6 h, 12 h, 24 h, and 48 h. Pools of 10 larvae (in each of 4 biological replicates) were washed out of the diet, quickly blotted on cellulose, transferred to 1.5 mL Eppendorf microvials (Thermo Fisher Scientific), weighed (fresh mass, FM), killed in liquid nitrogen, and stored at −80 °C until analysis. Samples for microarray analysis were taken at a single time, 24 h after cold stress. Pools of 10 larvae (in each of 3 biological replicates) were washed out of the diet, quickly blotted on cellulose, transferred to 1.5 mL microvials with 400 µL of RNA Extraction reagent (Amresco, Solon, OH, USA), cut to small pieces using fine scissors, and stored at −80 °C until analysis.

2.2. Metabolomics analysis

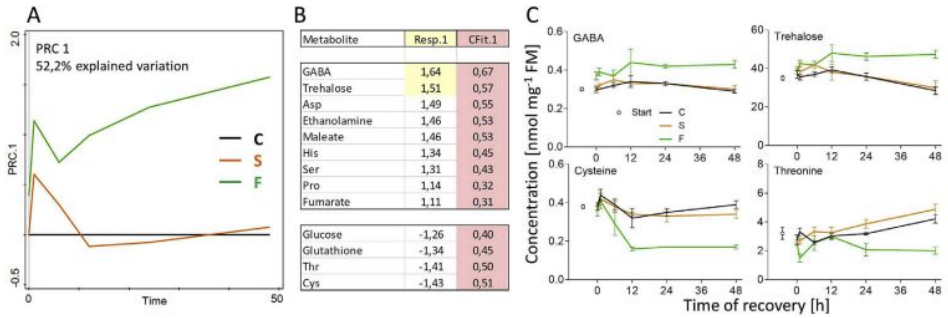
Larval metabolic composition was analysed using a set of chromatography methods coupled with mass spectrometry as described earlier (Štětina et al., 2018). Briefly, larvae were homogenized in a methanol:acetonitrile:water solution (volumetric ratio, 2:2:1) containing internal standards (*p*-fluoro-DL-phenylalanine, methyl α-D-glucopyranoside; Sigma-Aldrich, Saint Luis, MI, USA). Sugars and polyols were determined after *o*-methyloxime trimethylsilyl derivatization using a gas chromatograph (GC) with flame ionization detector GC-FID-2014 equipped with an AOC-20i autosampler (both from Shimadzu Corporation, Kyoto, Japan). Profiling of acidic metabolites was performed after treatment with ethyl chloroformate under pyridine catalysis and simultaneous extraction in chloroform (Hušek and Šimek, 2001). The analyses were conducted using a Trace 1300 GC combined with single quadrupole mass spectrometry (ISQ) (both from Thermo Fisher Scientific) and a liquid chromatograph Dionex Ultimate 3000 coupled with a high resolution mass spectrometer Q Exactive Plus (all from Thermo Fisher Scientific). All metabolites were identified against relevant standards and subjected to quantitative analysis using a standard calibration curve method. All standards were purchased from Sigma-Aldrich.

The results of metabolomics (summarized in the Supplementary Information, Dataset S1) were subjected to statistical analysis based on a constrained linear ordination method called redundancy analysis (RDA, Van den Wollenberg, 1977) using Canoco software, v5.04 (ter Braak and Šmilauer, 2012). RDA method computes ordination axes that summarize the effect of chosen predictors on the multivariate response data (here the metabolite concentrations). Concentrations of metabolites were first log-transformed (using a $ln(100*y + 1)$ formula) and then centered ($y'_i = y_i - \bar{y}$, where $\bar{y}$ is the mean of y_i values) and standardized ($y'_i = y_i/s_y$, where s_y is the standard deviation of y_i values). We used a specialized type of RDA called principal response curves (PRC), which focuses on time-dependent treatment effects on multivariate response data (Gaffney et al., 2018; Štětina et al., 2018; Van den Brink and ter Braak, 1999). Although the PRC analysis detects leading patterns in temporal metabolic profiles, its main difference from other similar tools, such as DBSCAN (Ester et al., 1996; Lehmann et al., 2018) is that the PRC analysis focuses on identification of *when* and *how* these profiles differ among different treatments (in our case C vs. S vs. F). Positions of treatment-time interaction terms on first and higher axes of the underlying partial RDA (with the main effect of time used as a covariate) are plotted against time, yielding one PRC curve for each experimental variant and axis. In addition, the temporal pattern of metabolic changes in the control variant was set as a reference value (0) and the temporal patterns of the cold-stressed treatments (S, F) therefore represent differences from the control.

2.3. Microarray analysis

Differential expression of *D. melanogaster* genes in response to cold stress was assayed using the FL003 *D. melanogaster* microarrays designed by the Flychip facility in Cambridge (http://www.flychip.org.uk/). Each microarray contains 18,240 spots, composed of transcript-specific oligonucleotides and controls developed by the International *Drosophila* Array Consortium (INDAC). A complete list of the 14,183 assayed transcripts represented on each microarray is shown in the Supplementary Information, Dataset S2. The methods were described in detail earlier (Koštál et al., 2017). Briefly, total RNA was isolated from larvae using Ribozol and treated with DNase I (Invitrogen, Carlsbad, CA, USA). First-strand cDNA was synthesized using Superscript III reverse transcriptase (Invitrogen). Second-strand DNA was synthesized using DNA Polymerase I and *E. coli* Ligase (both from Invitrogen) and the resulting DNA was purified with the Wizard SV Gel and PCR Clean-up-System (Promega, Madison, WI, USA). Next, one µg of double-stranded DNA was taken for Klenow labeling using Cyanine-5 dCTP (Cy5, Perkin Elmer, Waltham, MA, USA) and BioPrime DNA Labeling System (Invitrogen). Unincorporated dye was removed using the Illustra AutoSeq G-50 Dye Terminator Removal Kit (GE Healthcare). The Cy5-labeled DNA sample was blocked with sonicated salmon sperm DNA (Invitrogen), and used for hybridization on the microarrays. An automatic Hybridization Station HS4800 Pro (Tecan, Mannedorf, Switzerland) was used to perform all hybridization steps. All microarrays were scanned using the ScanArray Gx Microarray Scanner (PerkinElmer) at a resolution of 5 µm and the fluorescence was quantified using ScanArray Express v4.0.0.0004 software (Perkin Elmer).

The microarray signals passed multiple quality checks using R-based transcriptomics data preprocessor software RobiNA (Lohse et al., 2012). Using the same software, the log2 fluorescence values, normalized using quantile normalization (Supplementary Information, Dataset S2), were obtained from ScanArray data files. To minimize the risk of having transcripts with low fluorescence values showing high fold changes, no background subtraction was done. The log2 fluorescence values were first subjected to RDA statistical methods using Canoco software, v5.04 (ter Braak and Šmilauer, 2012) in order to assess clustering of transcriptional profiles according to treatment (C, S, F). Next, pairwise comparisons (S vs. C and F vs. C) were conducted using the limma R package (Ritchie et al., 2015). Genes with absolute fold changes > 1.5 fold and Benjamini and Hochberg adjusted P-value (FDRs, false discovery rates) < 0.1 were considered significantly differentially expressed (DE). Then, Gene Ontology (GO) and KEGG Pathway Enrichment Analysis were conducted on the DE genes using the R package ClusterProfiler (Yu et al., 2012). GO term categories with a minimum count of five significant DE genes and adjusted P-value < 0.1 were considered significantly enriched. KEGG pathways with adjusted P-value < 0.05 were considered significantly enriched. The Pathview R package (Luo and Brouwer, 2013) was then used to visualize the DE genes together with their fold change expression on KEGG pathway



Metabolite	Resp.1	CFit.1
GABA	1,64	0,67
Trehalose	1,51	0,57
Asp	1,49	0,55
Ethanolamine	1,46	0,53
Maleate	1,46	0,53
His	1,34	0,45
Ser	1,31	0,43
Pro	1,14	0,32
Fumarate	1,11	0,31
Glucose	-1,26	0,40
Glutathione	-1,34	0,45
Thr	-1,41	0,50
Cys	-1,43	0,51

Fig. 2. Summary of metabolic PRC 1-type response to cold stress in *Drosophila melanogaster* larvae (see section 2.2. for explanation of PRC analysis). (**A**) The metabolic changes in control (C) larvae were set as the reference value (0) and the temporal patterns (curves) of responses to supercooling (S) and freezing (F) represent a difference from the control. (**B**) Numeric results of PRC analysis for the most important metabolites– drivers of the PRC 1-type response. The parameter 'Resp.' describes the magnitude of response of a given metabolite in the statistical model; the parameter 'Cfit.' describes the proportion of explained variance by the statistical model (see Table S1 in Supplementary Information for complete results). (**C**) Examples of real temporal profiles in concentrations of four select metabolites most representative of the PRC 1-type response.

maps.

3. Results

3.1. Metabolome profiling

The results of metabolomics analysis of 35 different compounds are given in Dataset S1. Fig. S1 exhibits temporal profiles for three prominent amino acids: Asn, Gln, and Pro, which were present in relatively high concentrations in larvae, but did not rank very highly in the PRC analysis. The temporal profiles of Asn were practically equal in all three treatments. The profiles of Gln and Pro differed between treatments: the profile for frozen larvae deviated from the profiles for control and supercooled larvae, which were closely similar. The same major theme, a similarity of C and S profiles vs. deviation of the F profile, emerged from the comparison of the temporal trends in total (sum) concentrations of all targeted metabolites (Fig. S2), and also from the PRC analysis (presented in detail in Table S1).

The first set of PRC curves, explaining 52.2% of fitted variation in the metabolomics dataset, is shown in Fig. 2. The PRC 1 set clusters the metabolites exhibiting two clearly different temporal profiles: one similar for control and supercooled larvae vs. one for frozen larvae. In supercooled larvae there was an initial perturbation (until 12 h after cold stress), but the concentrations later matched the values observed in controls. In frozen larvae, these same metabolites showed a clear trend toward increasing deviation from controls over time (Fig. 2A). The list of important drivers of PRC 1-type response is shown in Fig. 2B (for more detail, see Table S1). Examples of temporal courses in the four most important metabolites according to PRC 1 set are given in Fig. 2C.

The other sets of PRC curves had much less explanatory power than PRC 1. The PRC 2 response (15.6% of explained variation) is depicted in Fig. 3 and the PRC 3 response (10.4% of explained variation) is depicted in Fig. S3. In supercooled larvae, the metabolites driving the PRC 2 response showed initial perturbations but finally their concentrations matched the values observed in controls. In frozen larvae, the PRC response drivers exhibited a transient peak at 12 h of recovery (for instance: alanine, lactate, and succinate in Fig. 3C), and/or a gradual decline far below the values of controls (for instance: glucose in Fig. 3C).

3.2. Gene transcript profiling

The results of microarray analyses of responses of 14,183 mRNA transcripts to cold stress (taken at 24 h after cold stress) are summarized in the Supplementary Information, Dataset S2. The transcriptomic responses significantly differed among treatments. The RDA found distinct clustering according to treatments (Fig. 4A), and suggested that the global transcriptomic responses to two different stresses, supercooling and freezing, share relatively little in common. The partial similarity between the two cold stress responses was detectable as a shift of both the S-cluster and F-cluster from the control cluster (C) along axis 2. Axis 2, however, explained only 16.9% of variation and, moreover, there were high within-treatment variations along the axis. The prevailing dissimilarity of responses to supercooling and freezing is suggested by their clear separation and shifts to opposite directions along axis 1 (37.1% explained variance).

The analysis of differential expression (for detailed summary see Supplementary Information, Tables S2A–D) identified 282 DE genes (227 upregulated; 55 downregulated) in response to supercooling, and 476 DE genes (256 upregulated; 220 downregulated) in response to freezing (both compared to control). Only 45 upregulated genes (10.3% of total) overlapped between responses to supercooling and freezing. The overlap was even smaller (2 genes; 0.7%) for downregulated genes (Fig. 4B, see Supplementary Information, Table S3 for a complete list of overlapping upregulated genes). These results confirmed the prevailing disparity of responses to supercooling and freezing.

The GO term enrichment analysis helped to identify biological processes in which the responses to supercooling and freezing either differ or overlap. Firstly, the GO terms related to chitin metabolism, cuticle development, and also the term 'response to toxic substance' were enriched among upregulated genes after both supercooling and freezing. The other terms that were enriched among upregulated genes differed between two stresses. Thus, the defense and immune responses were upregulated after supercooling, while a number of different pathways, dominated by amino acid catabolism, were upregulated after freezing (Fig. S4). Secondly, the terms linked to actin polymerization were enriched among downregulated genes after both supercooling and freezing. The processes related to muscle and cuticle development, cell division, phosphorylation, and phosphate metabolism were specifically downregulated after supercooling. A number of processes, dominated by response to oxidative stress and defense and immune responses, were specifically downregulated after freezing (Fig. S5).

The mapping of DE genes on KEGG pathways helped to further dissect the commonalities and differences in responses to supercooling and freezing. Because we set the cut-off criteria for significant response in a non-conservative (less stringent) way, the KEGG mapping sometimes identified pathways based on just one or two DE loci in a whole pathway. In the Supplementary Information, Tables S4A–D (where all

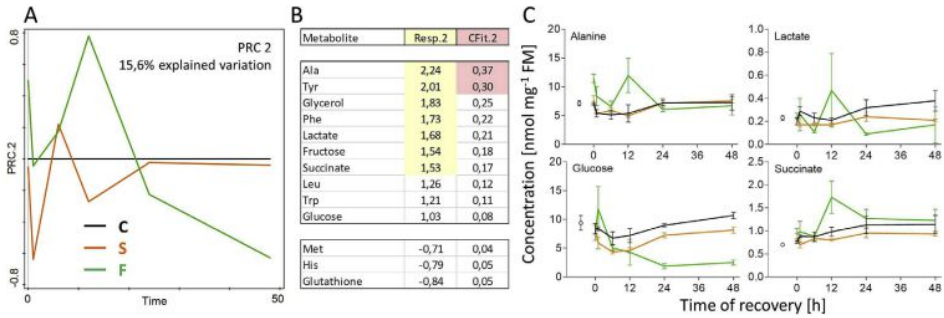


Fig. 3. Summary of metabolic PRC 2-type response to cold stress in *Drosophila melanogaster* larvae (see section 2.2. for explanation of PRC analysis). (**A**) The metabolic changes in control (C) larvae were set as the reference value (0) and the temporal patterns (curves) of responses to supercooling (S) and freezing (F) represent a difference from the control. (**B**) Numeric results of PRC analysis for the most important metabolites– drivers of the PRC 2-type response. The parameter 'Resp.' describes the magnitude of response of a given metabolite in the statistical model, the parameter 'Cfit.' describes the proportion of explained variance by the statistical model (see Table S1 in Supplementary Information for complete results). (**C**) Examples of real temporal profiles in concentrations of four selected metabolites most representative of the PRC 2-type response.

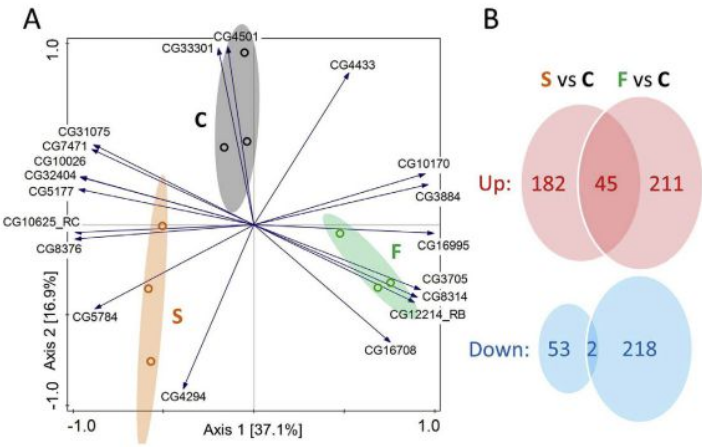


Fig. 4. Summary of the transcriptomic response in *Drosophila melanogaster* larvae to cold stress (see Tables S2, S3, and S4 in Supplementary Information for details). (**A**) The RDA analysis shows distinct clustering of control (C), supercooling (S), and freezing (F) treatments and also the loading vectors for selected mRNA transcripts [19 best fitting transcripts (fitting to statistical model > 97.5%) are shown; the transcript variant is explicitly mentioned only for variants RB, RC; all other transcript variants are RA). (**B**) Venn diagrams showing results of differential gene expression (DE) analysis. The numbers of significant DE genes (up- and downregulated) in pairwise comparisons S vs. C and F vs. C are shown.

results of KEGG analyses are summarized) we provide KEGG maps only for the most significant pathways. Firstly, the pathways 'Lysosome' (Fig. S6), and 'Toll and Imd signaling' (Fig. S7) were identified as most significantly upregulated in response to supercooling, while a great number of amino acid and other metabolites' pathways (mostly supported by a single locus) were found upregulated in response to freezing (Figs. S8–S10). Secondly, no downregulated pathway was identified in response to supercooling, while two pathways most downregulated in response to freezing were 'Lysosome' (Fig. S11) and 'Sphingolipid metabolism' (Fig. S12); we also added a KEGG map for 'Toll and Imd signaling' (Fig. S13). Although the Toll and Imd pathway signaling was not significantly downregulated in statistical terms (adjusted P-value = 0.11), its downregulation trend after freezing stress contrasted with the upregulation trend after supercooling stress.

Based on analysis of DE genes, GO enrichment, and KEGG mapping, we designed a schematic overview of the most important defense processes and effector molecules that were found differently regulated after supercooling and freezing (Fig. 5): namely (i) degradation of bacterial walls via Lysozymes (LysS, FBgn0004430; LysC, FBgn0004426); (ii) phagocytosis followed by lysosomal degradation of microbes via different acidic hydrolases such as cathepsins (Bace, FBgn0032049; CG33128, FBgn0053128), glycosidases (LAMAN: LManIII, FBgn0032066; HEX A/B: Hexo1, FBgn0041630), and lipase (LIPA: Lip3, FBgn0023495); and (iii) Toll and Imd signaling cascades that mediate response to microbial immune challenge and lead to the production of antimicrobial proteins (AMPs) such as Drosomycins (Drsl1, FBgn0052274; Drsl4, FBgn0052282), Attacins (AttA, FBgn0012042; AttB, FBgn0042581), Diptericins (DptA, FBgn0004240; DptB, FBgn0034407), Cecropins (CecA1, CG1365; CecA2, FBgn0000277), and Immune induced molecules (IM1, FBgn0034329; IM23, FBgn0034364).

4. Discussion

The larvae of *D. melanogaster* showed markedly different metabolic and transcriptomic responses to supercooling and freezing, both at −5 °C. The two cold stresses differed both in duration (the total exposure time at subzero temperature was 110 min and 230 min for S and F treatments, respectively), and in whether or not ice crystals formed inside the body. Freezing, in contrast to supercooling, is associated with osmotic dehydration of cells linked to a whole complex of deleterious consequences (for review, see Muldrew et al., 2004). Rather than directly comparing the responses to two different stresses, we will discuss responses to two stresses more or less independently focusing on specific questions: (i) what, if anything, the larvae do in order to survive

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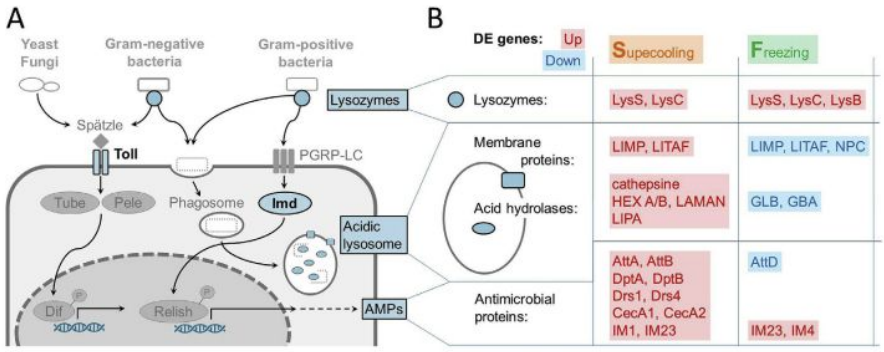

Fig. 5. Schematic overview of (**A**) Toll and Imd signaling cascades that mediate the response to microbial immune challenges and lead to stimulation of defense response effector molecules such as Lysozymes, components of acidic lysosomes, and antimicrobial peptides. These processes and effectors were all upregulated after supercooling according to DE analysis, GO term enrichment analysis, and KEGG pathway mapping analysis (for more details see Figs. S7 and S13). (**B**) A list of effector molecules of the defense response that were transcriptionally either upregulated (pink rectangles) or downregulated (blue rectangles) in response to supercooling and freezing (see sections 3.2 and 4.2. for explanations). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

after supercooling (S vs C comparison); (ii) what characterizes the survivor (but mostly the destined-to-die) larvae exposed to freezing (F vs C comparison).

4.1. Response to supercooling indicates tolerance of injury and active repair processes

Considering high survival after supercooling (> 95% survival to adult stage) and relatively weak sublethal effects (a reduction of reproductive capacity of survivor females to 75.7% of controls), a question emerged as to whether the temperature of −5 °C was stressful at all. In other words, we were not able to decide conclusively in our parallel study (Koštál et al., 2019) whether the quiescent larvae *resisted* the supercooling injury (no injury occurred), or *tolerated* the incurred injury and/or actively repaired it. Detailed analysis of metabolic and transcriptomic changes indicated that the response to supercooling in quiescent larvae of *D. melanogaster* differed from controls. The supercooled larvae had to cope with cold-induced perturbation of metabolic homeostasis and recruited specific transcriptomic responses, likely at an energetic cost. These results suggest that the supercooling treatment is likely stressful, and the response to supercooling is, at least partially, of tolerance type.

Metabolomics analysis revealed that supercooling was associated with a transient initial perturbation of metabolism. During the first 12 h of recovery after supercooling stress, various metabolites showed rapid fluctuations in concentration that were not seen in controls. Nevertheless, the metabolic profiles of supercooled larvae returned to 'normality', and were strikingly similar to profiles taken in control larvae screened 24 h or 48 h after cold stress. These results indicate that supercooling was not simply resisted but rather perceived as a sort of stress resulting in misbalance of the metabolic network. As the effect on the larval metabolic network was only transient, the results further suggest that the feedback-loop mechanisms were sufficiently robust to return the challenged organism back to metabolic homeostasis within 24 h. In accordance with our observations, various metabolic perturbations caused by cold stress were observed earlier in adults of *D. melanogaster* (Colinet et al., 2012; Overgaard et al., 2007), and it was shown that metabolic networks are more robust (less responsive to cold shock) in the lineages of *D. melanogaster* selected in laboratory for increased cold hardiness (Williams et al., 2014).

Microarray analysis further corroborated the distinction between supercooled and control larvae. Of the 282 transcripts differentially expressed in response to supercooling (2.0% of the total 14,183 mRNA transcripts analysed), 227 were up- and 55 were downregulated. The downregulation response was relatively weak and the GO term enrichment analysis suggested, perhaps not surprisingly, that various developmental processes (e.g. muscle cell development, cuticle development, cell division, developmental maturation, cellular component biogenesis, and organelle assembly) were downregulated. In addition, the GO terms related to actin filament organization were enriched among the downregulated genes (in response to both supercooling and freezing). Cytoskeletal stabilization and rearrangement has emerged as a recurring theme linked to both insect cold acclimation (Kim et al., 2006; Des Marteaux et al., 2017, 2018a,b), and cold injury repair (Kayukawa and Ishikawa, 2009; Teets et al., 2012), particularly with regards to defense of polymerized actin. KEGG mapping failed to identify any enriched pathway downregulated in response to supercooling. In contrast, the upregulation response to supercooling exhibited the single strongest unifying theme: immune defense, which was reflected in both GO term enrichment and KEGG mapping analyses (Fig. 5).

4.2. Response to supercooling is dominated by transcriptional upregulation of immune response pathways and antimicrobial peptides

The GO term enrichment and KEGG mapping analyses revealed the 'Defense response to Gram-positive bacterium' (GO:0050830), 'Toll and Imd signaling pathway' (KEGG pathway dme04624), and 'Lysosome' (KEGG pathway dme04142) as the most significantly upregulated processes in response to supercooling in larvae of *D. melanogaster* (Fig. 5A). For instance, the genes encoding Spheroide (FBgn0030774) and Spätzle processing enzyme (SPE, FBgn0039102) were upregulated in response to supercooling. Spheroide and SPE are serine proteases, and SPE integrates responses to various infections as well as cellular damage (Buchon et al., 2014). SPE is activated by proteases released from virulent fungi and bacteria (Gottar et al., 2006), by signals released from necrotic cells (Ming et al., 2014), and also by microbial cell wall components (Gobert et al., 2003). Activated SPE cleaves Spätzle proteins, leading to Toll pathway activation in the fat body and haemocytes (Buchon et al., 2009). Activated Toll pathway, in turn, leads to the systemic production of antimicrobial proteins (AMPs), with antifungal peptide Drosomycin as a principal target (Valanne et al., 2011). Accordingly, KEGG mapping and DE analysis identified significant upregulation in a whole set of genes coding for effector molecules of

immune response such as Lysozymes, various antimicrobial proteins, and different acidic hydrolases (Fig. 5B), which may help to digest macromolecules after phagocytosis of microbial cells and formation of acidic lysosomes (Gao et al., 2017). Such complex transcriptional response suggests that immune response pathways are activated upon supercooling stress.

It has been reported earlier that cold stress may induce transcriptional immune response in adults of *D. melanogaster* (Salehipour-Shirazi et al., 2017; Sinclair et al., 2013; Zhang et al., 2011). The relationship between potential and realized immune response is not, however, straightforward. The estimates of immunity based on gene expression (potential) need not match the realized ability to defend against pathogens (Fedorka et al., 2007). Indeed, some authors reported that cold stress increases resistance to fungal infection (Le Bourg et al., 2009), while others showed that realized immunity actually decreases after cold shock in *D. melanogaster* (Salehipour-Shirazi et al., 2017). Analysis of the cold-related immunity in different insects revealed a high level of idiosyncrasy (Ferguson and Sinclair, 2017). It has been shown that low temperatures can both suppress and activate immune responses in ectothermic animals depending on species, overwintering habitat, level of cold stress, type of pathogen, and also depending on which particular metric of immune response is measured (Ferguson et al., 2018). Therefore, our results on complex transcriptional activation of immune response pathways after supercooling in larvae of *D. melanogaster* will require rigorous functional validation at the level of realized immunity.

At this stage of research we are unable to conclusively decide on neither the triggering signals nor the adaptive meaning of the observed transcriptional activation. The transcriptional response could be triggered by microbial signals, perhaps coming from Lysozyme-mediated degradation of bacterial walls of gut microbiota (Douglas, 2015; Engel and Moran, 2013; Moghadam et al., 2018). Alternatively, the response may be triggered by signals released from cold-damaged cells (Ming et al., 2014), or non-specifically by low temperature via broader activation of multiple defense responses, for instance to the occurrence of partially denatured/misfolded proteins (Ananthan et al., 1986; Kaunisto et al., 2016; Štětina et al., 2015; Wallin et al., 2002). The response could be adaptive (helping to cope with increased microbial attack after cold stress), or prophylactic (preparing the organism for the case of increased microbial attack), or even maladaptive (consuming energy when no increase in microbial attack occurs). One way to assess adaptiveness of the immune response for survival after cold stress would be to compare the cold stress responses between wild and mutant or RNAi lineages of *D. melanogaster* deficient in Toll, Imd and other relevant pathways. An alternative way would be to expose wild-type animals to different infection loads and observe how the survival and fitness of survivors are influenced after cold stress.

4.3. Response to freezing includes downregulation of immune response pathways

The exposure to freezing at −5 °C was clearly stressful to larvae of *D. melanogaster* as only approximately one in ten treated larvae survived to adulthood. Nevertheless, most larvae were still alive 24 h after the stress and exhibited delayed mortality (Koštál et al., 2019). In addition, the reproductive capacity of freezing-stress-survivor females was reduced to 47.9% relative to controls (Koštál et al., 2019). Hence, the described freezing-induced metabolic and transcriptomic patterns will likely combine the responses linked to upcoming mortality (unsuccessful repair) and to survival (tolerance and partial repair).

Metabolomics analysis showed that the perturbation of metabolic networks was deeper in frozen larvae relative to those that were supercooled and, importantly, this perturbation was irreversible in most frozen larvae. The PRC curve analyses suggested that the initial phase of recovery (until 6 h after stress) was associated with similar disturbance trends in supercooled and frozen larvae. Afterwards, however, the frozen larvae deviated increasingly from controls and supercooled larvae as recovery time progressed. Most frozen larvae died during the interval of 24–96 h after the stress and their mortality was preceded by a peak (at 12 h of recovery) of anaerobic end-products such as alanine, lactate, and succinate. At the same time (12 h), peaks of some amino acids (Gln, Phe, Pro, Ser, Thr, Trp, Tyr, Val) were observed in frozen but not supercooled larvae. This result corresponds well with the results of GO term enrichment analysis (returning 'Amino-acid catabolic process' as the most significantly upregulated process) and KEGG mapping (returning many different amino acid metabolisms among the upregulated pathways). We interpret these results as signifying gradual failure of life functions, including respiration and aerobic energy production (6–12 h after the stress), prevailing degradation of macromolecules (12–24 h after stress), and death (24–96 h after stress).

Some elements of transcriptomic responses were shared between supercooled and frozen larvae. Although only 45 DE genes (less than 10% of all DE genes) were shared, the list contained 11 genes to which different defense functions are broadly ascribable, including those coding for Lysozymes LysS, LysC, and Immune induced molecule IM23. Another 15 genes on the list are broadly related to degradation of macromolecules (proteases, glucosidases, lipases), which can also be regarded as a part of the defense or injury-repair response. In contrast to supercooled larvae, the frozen larvae showed a *down*regulation of Toll and Imd signaling pathways. Thus, it seems that both supercooling and freezing triggered some defense mechanisms, but the response to freezing was less complex or not complete, perhaps interrupted and overwhelmed by death-related processes (see downregulation of elements in several death-related pathways in frozen larvae: 'Autophagy', 'Apoptosis', and 'Sphigholipid metabolism'). Due to the nontrivial relationship between transcriptional activation (potential) and realized immune response (Ferguson et al., 2018; Ferguson and Sinclair, 2017), we cannot decide (based on our current results) whether relatively weak transcriptional activation of the immune response was a consequence or a cause of freezing injury and developing mortality in frozen larvae.

Collectively, the stimulation of immune response pathways appears as a strong candidate tolerance mechanism for coping with supercooling stress in larvae of *D. melanogaster*. Here we described complex transcriptional activation of innate immunity: from Lysozyme-mediated degradation of bacterial cell walls, recognition of pathogens' signals, through phagocytosis and lysosomal degradation, Toll and Imd signaling pathways, to upregulation of genes coding for effector molecules – antimicrobial peptides. It will require further effort, however, to learn whether this elevation of immune potential is reflected in an adaptive increase of realized immunity.

Conflicts of interest

The authors have declared that no competing interest exists.

Data accession

Microarray raw data were deposited in the ArrayExpress database at EMBL-EBI (www.ebi.ac.uk/arrayexpress) under accession number E-MTAB-7401.

Acknowledgements

This work was supported by the Czech Science Foundation (grant GAČR, 16-06374S). We thank Jaroslava Korbelová, Irena Vacková, Anna Heydová, Iva Opekarová, and Helena Zahradníčková (all from Biology Centre, CAS) for assistance with insect rearing, sample preparation, and analyses. Lauren Des Marteaux (Biology Centre, CAS) commented on an early version of the paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ibmb.2019.01.006.

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8.3. Paper III

Štětina, T., Des Marteaux, L.E., Koštál, V. Insect mitochondria as targets of freeze-induced injury. (manuscript in preparation)

The abstract of the paper is present in the current form submitted to Proceeding of Royal Society B, at the moment the paper is subjected to the first round of revision.

Insect mitochondria as targets of freezing-induced injury.

Štětina T., Des Marteaux L.E., Koštál V.

Abstract

Many insects survive internal freezing, but the great complexity of freezing stress hinders progress in understanding the ultimate nature of freezing-induced injury and the mechanisms of freeze tolerance. Here we use larvae of the drosophilid fly, *Chymomyza costata* to produce phenotypic variants differing in freeze tolerance and assess the influence of phenotype on mitochondrial responses to freezing stress. Using transmission electron microscopy, we show that fat body and hindgut mitochondria swell, and occasionally burst, upon exposure of the non diapause (freeze-sensitive) phenotype to lethal freezing stress. In contrast, mitochondrial swelling is not observed in the diapausing, cold-acclimated (freeze-tolerant) phenotype exposed to the same stress. Mitochondrial respiration analysis revealed that fat body and muscle mitochondria of the freeze-sensitive phenotype significantly decrease oxygen consumption upon lethal freezing stress, while mitochondria of the diapausing, cold-acclimated phenotype do not lose respiratory capacity upon the same stress. Further, we found that citrate synthase and mitochondrial enzymatic complexes involved in the electron transport chain and oxidative phosphorylation retain partial capacity to function even in lethally-injured freeze-sensitive larvae. These results suggest that proteins (enzymes) 'survive' freezing stress, which is otherwise lethal at the organismal level. We hypothesize, that mitochondrial swelling and bursting results from permeability transition of inner mitochondrial membrane and loss of its barrier function, which causes osmotic influx of cytosolic water into the matrix. The phenotypic transition to diapause and cold acclimation is associated with adaptive change that protects the inner mitochondrial membrane against permeability transition and subsequent mitochondrial swelling. Accumulation of high concentrations of proline and other cryoprotective substances might be a part of adaptive change as we have shown that freezing-induced mitochondrial swelling was abolished by feeding the freeze-sensitive phenotype larvae on a proline-augmented diet.

9. Summary of results and conclusions

I. Recovery from cold stress – *Chymomyza costata*

The study captured major physiological processes occurring in larvae of highly freeze-tolerant drosophilid fly, *C. costata* after the exposure to various intensities of cold stress: relatively harmless supercooling to -10°C vs. sublethal freezing to -30°C vs. partially lethal cryopreservation at -196°C. The findings are novel and original since the post-stress processes, including repair of injury, have been mostly neglected so far in insect cold hardiness literature. We observed almost immediate recovery of respiration, including the re-establishment of transmembrane ion potentials, even after exposure to freezing and cryopresevation in LN2. The global metabolomic and transcriptomic analyses revealed significant differences between control and supercooled larvae, which was not expected as the supercooling treatment was considered practically harmless (showing no effect on larval survival or timing of further development). In contrast, the differences between supercooled and frozen larvae were relatively weak, showing that both stresses may have similar targets and stimulate similar repair processes, which again was not expected, as the physicochemical consequences of supercooling (low temperature) and freezing (low temperature plus cell dehydration plus presence of ice crystals) are drastically different. Next, metabolomic and transcriptomic profiles of cryopreserved larvae (alive but moribund) differed significantly from frozen larvae (repairing, destined to survival). These differences provided a hint of the mechanisms of freeze injury and its repair. Several lines of evidence pointed toward mitochondria as potential targets of freeze injury in cryopreserved larvae: the numerous diversions of glycolytic flux from TCA toward accumulations of side-branches derivatives such as fructose, myo-inositol, sorbitol, alanine, sarcosine, and glycine; the decreases of citrate, aconitate, and ketoglutarate levels suggested partial blockade of pyruvate entry to, or early steps of, the TCA cycle in the mitochondrial matrix; the accumulation of ornithine indicate re-routing of mitochondrial catabolic pathways for excess amino acids (proline, glutamine) from TCA (partially blocked) toward ornithine; the failure to upregulate 116 specific sequences, mostly involved in energy metabolism, that might be important for successful repair of cold injury and averting the delayed mortality during recovery from freezing stress. There were only three sequences upregulated specifically in LN2-treated larvae. Two of them, hsp22 (small heat shock protein) and Atg8a

(autophagy-specific protein) are again tightly linked to mitochondrial survival and stability.

II. Recovery from cold stress – *Drosophila melanogaster*

The study provided an insight into physiological processes occurring during recovery from two physiologically distinctive cold stresses – supercooling and freezing at -5°C – in relatively freeze sensitive organism, *D. melanogaster*. The metabolomics profiling revealed significant perturbations of homeostasis during first hours after exposure to both supercooling and freezing stresses but only the larvae of supercooled variant returned to pre-freezing pattern, whereas metabolite profiles of frozen larvae further deviated. The significant accumulations of anaerobic products, such as alanine, lactate or succinate, after freezing stress, pointed toward a failure of mitochondrial aerobic respiration. The microarray analysis of transcriptomic profiles confirmed different patterns of response between supercooled and frozen larvae. Supercooling resulted in highly complex activation of innate immunity pathways, covering genes coding for lysozymes, through members of Toll and Imd signaling pathways, to production of different anti-microbial peptides. In contrast, freezing stress failed to activate immune pathways or even resulted in significant downregulations of some of them.

III. Mitochondria as targets of low temperature-induced injury

Earlier studies suggested that energy metabolism and mitochondrial functions are strongly affected by temperature stress. In this study, mitochondrial responses to cold and freezing stress were directly compared in relatively freeze-sensitive (non-diapause, warm acclimated) and two freeze-tolerant acclimation variants (proline-fed and diapause, cold acclimated) of *C. costata* larvae. The acclimations appeared to have relatively small effects on mitochondrial counts, morphology and functions in fat body and hindgut tissues. Relatively mild cold stress (either supercooling or freezing at -5°C) also had no or little impact on mitochondria. The severe freezing stress (-30°C), however, resulted in mitochondrial swelling, bursting and loss of respiratory function in the freeze sensitive phenotype. In contrast, the mitochondria of freeze-tolerant phenotypes exposed to the same stress retained normal morphology and functionality. Surprisingly, isolated components of the energy metabolism, such as citrate synthase, complexes of electron transfer chain and ATP synthase retained at least partial functionality even in lethally injured (dead) freeze-sensitive larvae. This result challenges the widespread paradigm considering the proteins (enzymes) as

important primary targets of freezing-induced injury. Related to this paradigm, accumulated metabolites such as proline and trehalose, are believed to protect the proteins (enzymes) from loss of structure (denaturation and aggregation) upon freezing stress. In our study, we suggest that biological membranes, including inner mitochondrial membrane (IMM), might be even more important targets of freeze injury than proteins. We hypothesize that a sudden loss of IMM barrier function – a permeability transition, might be one of the ultimate causes of freeze injury. The freeze-tolerant phenotypes can prevent/avoid the IMM permeability transition, perhaps also thanks to cryoprotection of membrane integrity exerted by proline and trehalose.

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Low-temperature injury in insect tissues and mechanisms of its repair
Ph.D. Thesis Series, 2020, No. 14

Printed in the Czech Republic by Typodesign
Edition of 20 copies

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