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Insect overwintering: physiological and biochemical adaptations to low temperatures

Ph.D. Thesis

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▨ Annotation

Complex experimental data on insect adaptation for survival at low temperatures, with special reference to overwintering in temperate climate zone, are presented in this thesis. The cold tolerance strategies were examined in four species: the codling moth (*Cydia pomonella*), the bark beetle (*Ips typographus*), the red firebug (*Pyrrhocoris apterus*), and the fruit fly (*Drosophila melanogaster*). Two of these species, *C. pomonella* and *I. typographus*, are serious pests and knowledge on their overwintering biology is of great importance for development of pest management programs. Numerous physiological and biochemical parameters, such as supercooling point, thermal hysteresis between melting and freezing points, survival in supercooled and frozen states, osmolality of hemolymph, water and energy reserves, and detailed metabolomic composition of hemolymph and tissues were studied during cold season in the field-collected animals or analyzed in laboratory-acclimated animals in order to assess the insects' capacity for winter survival.

▨ Declaration [in Czech]

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■ List of papers and author's contribution

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

I.   Košťál V., Doležal P., **Rozsypal J.**, Moravcová M., Zahradníčková H., Šimek P. (2011). Physiological and biochemical analysis of overwintering and cold tolerance in the spruce bark beetle, Ips typographus. Journal of Insect Physiology 57, 1136-1146. (IF: 2.236)

   Jan Rozsypal participated in sample preparations, was responsible for measurement of SCP, osmolality and thermal hysteresis, and revised the manuscript.

II. Košťál V., Renault D., **Rozsypal J.** (2011). Seasonal changes of free amino acids and thermal hysteresis in overwintering heteropteran insect, Pyrrhocoris apterus. Comparative Biochemistry and Physiology 160A, 245-251. (IF: 2.235)

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III. Košťál V., Korbelová J., **Rozsypal J.**, Zahradníčková H., Cimlová J., Tomčala A., Šimek P. (2011). Long-term cold acclimation extends survival time at 0°C and modifies the metabolomic profiles of the larvae of the fruit fly Drosophila melanogaster. PLoS ONE 6(9): e25025 (IF: 4.351).
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   Jan Rozsypal participated in sample preparations, SCP measurement, and revised the manuscript.

IV. **Rozsypal J**, Košťál V, Zahradníčková H and Šimek P (2013) Overwintering strategy and mechanisms of cold tolerance in the codling moth (*Cydia pomonella*). PLoS ONE. (Article in press)

   Jan Rozsypal conceived and designed the experiments, conducted sampling, sample preparations, measurement of SCP, osmolality, thermal hysteresis, energy reserves, preparation of survival assays, data assembly, statistical analysis, and wrote the manuscript.

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Contents

1. Introduction

Insects represent one of the most successful groups of organisms, which have evolved on Earth. They inhabit almost every environment from the tropics to the polar regions. Especially those species inhabiting higher latitudes have evolved numerous adaptations to the seasonally changing environmental conditions. Low temperatures in winter or the dry season in tropics represent the most serious dangers, which insect must deal with.

This chapter should introduce the reader to current knowledge of insect adaptation to changing environmental conditions concentrating on cold tolerance and overwintering. Strategies and mechanisms involved in overwintering will be outlined and several examples will be presented. The mechanisms involved in overwintering are the main subject of my interest. My thesis focuses mainly on numerous physiological and biochemical parameters which may play a key role in overwintering success. The overwintering larvae of the codling moth (*Cydia pomonella*) served as model species for my studies. Other species involved in my thesis are the red firebug (*Pyrrhocoris apterus*), the spruce bark beetle (*Ips typographus*) and the fruit fly (*Drosophila melanogaster*).

1.1. Diapause

Environmental conditions on Earth change seasonally. Periods favorable for growth, development and reproduction are limited to a specific time of year (summer). Seasonal changes become more apparent with increasing latitude. While the changes are very mild or negligible in the tropical zones, they become more apparent in temperate zones and reach their maximum in the polar zones. In the temperate zone, growth, development and reproduction of ectotherms, including insects, are usually restricted to favorable conditions of summer. On the contrary, winter conditions such as low temperatures influence life functions and rate of metabolic processes and pose a threat of chilling or freezing injury and also a risk of desiccation. Seasonal changes have a cyclic annual and thus predictable pattern. In response to the predictable nature of these changes many insect species have evolved a form of dormancy – diapause.

Diapause can be considered an alternative developmental pathway characterized by many significant changes. These changes involve the halt of development or reproduction, alterations to the developmental program (to various extent), suppression and changes in metabolism and gene expression, which is behind these changes. Diapause occurs during a specific stage of development, which is species specific and results in deep change of phenotype (Tauber et al., 1986a,b; Denlinger, 2000, 2002; Macrae, 2005). For example, the silkmoth (*Bombyx mori*) enters diapause as an early embryo (Yamashita, 1996), the codling moth (*Cydia pomonella*) diapauses in the last larval stage (Ashby and Singh, 1990), the cabbage butterfly (*Pieris brassicae*) in the pupal stage (Pullin, et al. 1991), and the European peacock butterfly (*Inachis io*) as an adult (Dvořák et al., 2009). The diapausing stage may take very different forms, from various immobile stages such as diapausing embryo or pupa, which do not accept any food, to fully mobile feeding larvae or adults (Košťál, 2006).

Diapause is understood as a dynamic process consisting of several more or less distinct phases: induction, preparation, initiation, maintenance, termination and post-diapause quiescence (Košťál, 2006). The **induction phase** occurs during a specific developmental stage, when the environmental cues are perceived and transduced into switching the ontogenetic pathway from direct development to diapause development. The **preparation phase** takes place in species, which have a period of direct development between induction and initiation phases, but some preparations (physiological or behavioral) may take place as well. Direct development ceases during the **initiation phase** and metabolism is suppressed. Mobile diapausing stages may continue to feed and seek a suitable overwintering site. The developmental arrest persists during the **maintenance phase**, even if the conditions are favorable for development. Metabolic rate is low and relatively constant. Sensitivity to diapause-terminating conditions gradually increases. **Termination of diapause** occurs when some specific changes in the environmental conditions stimulate the decrease of diapause intensity to its minimum level and thus synchronize individuals within the population. By the end of the termination phase, a physiological state allowing resumption of direct development (if the conditions permit) is reached. After diapause is terminated, the insects may remain in a state of **post-diapause quiescence** which is maintained directly by environmental factors such as temperature (for more detail on diapause phases see Košťál, 2006).

Diapause may be obligatory or facultative. Species with obligatory diapause enter diapause regardless of the environmental conditions. Diapause is an integral part of their ontogenesis. However, most species enter facultative diapause, which is induced by specific environmental stimuli. Most of the insect species have evolved an ability to perceive and respond to specific environmental stimuli (so called token stimuli) that signal the upcoming seasonal changes (Tauber et al., 1986a). The most common token stimulus (or signal) is shortening day-length in late summer and early autumn. Day-length (or, more precisely, night-length in most cases) is perceived by a combination of visual and non-visual light receptors, processed in the brain and transduced to changes in a hormonal signal, which is responsible for changes in physiology, biochemistry, morphology and behavior (Yin and Chippendale, 1976; Bell, 1994; Denlinger, 2000, 2002). Although the photoperiod is considered the major signal, there are other cues such as temperature, quality of food, social and ecological interactions, intraspecific or interspecific, which have an influence on diapause incidence (Steinberg et al., 1992a,b; Košťál, 2006).

Soon after initiation, diapause is maintained even if diapausing insects experience conditions favorable for growth and reproduction (Tauber et al., 1986a). Diapause thus prevents insects from resuming development prematurely, for example when a short period of favorable conditions occurs during autumn. The process of diapause termination is still not completely understood and certain disagreements about this subject remain to be resolved. While in some insects kept under constant laboratory conditions the termination of diapause is spontaneous, some other insects kept under constant conditions maintain diapause until they die. In the field diapause termination is initiated when some environmental conditions or stimulus or combination of stimuli occur. As individuals of the same population enter diapause during different parts of the year, each of them maintains diapause for different period of time. Diapause is then maintained until the period of adverse conditions come, which serves as synchronizing stimulus and also prevents premature termination of diapause. During the termination phase the intensity of diapause gradually decreases, until the resumption of direct

development is enabled (Košťál, 2006). Some authors suggest low temperatures to be the cue which initiate diapause termination, but some other authors consider different cues like high temperatures, photoperiodic signal or contact with water (moisture) (for review see Tauber and Tauber, 1976; Hodek, 2002; Košťál, 2006).

Diapause enables insects to survive through periods unfavorable to development and reproduction such as periods of cold or drought and it also synchronizes activities of the individuals within the population. In some species, diapause seems to directly influence the resistance to adverse environmental conditions, while in some other species the relationship between diapause and resistance to environmental conditions is not so obvious. Some insect species become cold resistant (cold-hardy) as they enter diapause, but other species need a period of cold acclimation to reach a certain level of cold hardiness (for more details on cold acclimation see section 1.3.4.). The relationship between diapause and cold hardiness may vary from purely coincidental to tightly linked (for review see Denlinger, 1991; Pullin, 1996; Hodková and Hodek, 2004).

1.2. Overwintering strategies

Insect species inhabiting the temperate zone must deal with different conditions during different parts of the year. Temperatures in summer may reach high values; there may be periods without rainfall resulting in low humidity. Such conditions may pose a threat for the relatively small insect body in terms of desiccation. Insects may encounter somewhat similar situation in winter when a combination of low temperature, absence of liquid water / presence of ice in the environment and low absolute humidity of the air may cause substantial loss of body water.

Most obviously, sub-zero temperatures during winter represent a risk of formation of ice within the insect bodies. While some insects tolerate extracellular freezing, intracellular freezing is considered incompatible with life processes (Storey and Storey, 1988). In theory insects have three options for dealing with seasonally low temperatures: to migrate out of the cold region, to regulate body temperature or to tolerate low body temperatures.

Migration over long distances, though not widespread among insects, represents one of the possible strategies. For instance, Monarch butterflies (*Danaus plexippus*) migrate from the USA to Mexico (Urquhart and Urquhart, 1976; Brower, 1995). Overwintering strategy of many insect species includes migration over short distances. They avoid low temperatures by vertical migration into the soil or selection of protected hibernacula (Lee, 1989). Substances such as litter provide an adequate protection against harsh winter conditions and minimize the risk of cold injuries. Species originating from sub-tropical and tropical zones often search for their hibernacula in human residences (Bale, 1996). Selection of a suitable site is thus vital for many insect species for winter survival (Danks, 2006).

Another strategy is to **regulate body temperature**. In insects, thermoregulation is feasible only at superorganism level of some colonial and social insect species. Honey-bees (*Apis spp.*), which probably represent the best known example of this strategy, maintain a constant hive

temperature of approximately 35°C (20°C to 35°C in winter) by behavioral and physiological activities of the colony (Heinrich, 1981; Southwick and Heldmaier, 1987).

The last, but clearly most widespread option is to **tolerate low body temperature**. Many insect species cannot evade the exposure to low temperatures, thus they have evolved a number of physiological and biochemical adaptations, which we refer to as cold hardiness.

1.3. Cold hardiness

Temperature influences every aspect of insect's life, from rate of biochemical reactions which are well known to be temperature dependent, to growth, development and reproduction. Cold hardiness refers to the ability of an insect (or any organism) to survive exposure to low temperatures (Salt, 1961; Danks, 1978; Zachariassen, 1985; Lee, 1989). Physiological mechanisms of cold hardiness (cold tolerance) are the focus of this thesis.

1.3.1. Classes of cold hardiness

Insects can be divided into two major categories, according to their strategy for survival in the cold: "**freeze-avoiding**" and "**freeze-tolerant**" (Lee, 1991; Sinclair et al., 2003). Freeze-avoiding insects cannot survive ice formation in their body fluids and often die well above temperature of crystallization of their body fluids (supercooling point, SCP; see section 1.3.3. for more information). Freeze-tolerant species can survive partial freezing of their body fluids (Lee, 1989; Bale, 1993; Renault et al., 2002). Although these two strategies are different in principle, they share several similarities and some species are capable of switching from one strategy to the other (for example: Horwath & Duman, 1984; Fields and McNeil, 1986). Because of apparent deficiency of only having two categories, some authors attempted to establish a new system of classification. Bale (1993, 1996) proposed a new system of classification defining five categories of cold hardiness: 1) Freeze tolerance, 2) Freeze avoidance, 3) Chill tolerance, 4) Chill susceptibility and 5) Opportunistic survival.
Sinclair (1999) proposed a new system of classification consisting of four categories within the "freeze tolerance" category based on SCP and the point of lower lethal temperature: 1) Partial freezing tolerance, 2) Moderate freezing tolerance, 3) Strong freezing tolerance and 4) Freezing tolerance with low SCP. Unfortunately, the systems of classification of cold hardiness suffer from the same flaw as almost all such systems. No matter how many categories are defined, an exception breaking the rule can be always found. This inconsistency may be the main reason that, despite efforts for more thorough sorting, the old division into two categories is still widely used in literature.

The main issue insects must deal with at subzero temperatures is the phase transition of water from liquid to solid. Insects have fundamentally five options how to deal with formation of ice: **1) Supercooling** represents the most widespread strategy, when formation of ice is to be avoided (Lee et al., 1996) (for more information on supercooling see section 1.3.3.). **2) Extracellular freezing** represent another strategy widespread in insects. Opposite to

supercooling, the formation of ice is initiated at mild sub-zero temperatures (Storey and Storey, 1988). **3) Intracellular freezing**, although usually considered lethal (Zachariassen, 1985; Storey and Storey, 1988), is tolerated at least in some animals. Though this strategy has not been described in insects yet, some authors reported that isolated fat body cells of diapausing *Eurosta solidaginis* can survive intracellular freezing (Salt, 1962; Davis and Lee, 2001). There is also one known example of survival of intracellular freezing at the organism level. A nematode *Panagrolaimus davidi* can survive extensive intracellular freezing (Wharton and Ferns, 1995). **4) Cryoprotective dehydration** was reported in several arctic collembolans (Holmstrup and Sømme, 1998; Sørensen and Holmstrup, 2011). The potential danger of formation of ice is avoided by removing most of the body water. **5) Glass transition (vitrification)** occurs when liquid become solid at temperatures usually far below the normal freezing point, but without the formation of crystalline phase (MacFarlane, 1987). A glass is a solution of high viscosity, which prevents all physical and chemical reactions that require molecular diffusion, including ice nucleation (Danks, 2000). Some authors reported vitrification to be an essential part of the survival strategy of anhydrobiotic invertebrates (Sakurai et al., 2008; Hengherr et al., 2009) and some deeply supercooled arctic beetles (Sformo et al., 2010).

1.3.2. Effects of low temperatures

Low temperatures can be defined in various ways because it may cover a wide range of temperatures. Low temperature can be understood as any temperature below the threshold for activity, growth and development, however, more strictly, it is a temperature which has a negative impact on insect physiology, causes some sort of injury, and, consequently, a loss of fitness and/or mortality (Lee, 1991). The lowest temperature that causes no significant mortality during prolonged exposure to cold is referred to as upper limit of cold injury zone (ULCIZ) (Nedvěd, 1998; Nedvěd et al., 1998). The range of low temperatures is species-specific and depends on the extant physiological state of the insect. Even temperate insects may die when exposed to temperatures considerably above 0°C when not properly acclimated. Most attention is focused on sub-zero temperatures and their effects on insect organism.

Insects exposed to low temperatures may suffer a cold injury. Mechanisms of cold injuries are still not completely understood because of their complexity (low temperature influences biological processes at all levels). Cold injury can be divided into two main categories: freeze injury and chill injury (Storey and Storey, 1988). Freeze injury occurs as a result of ice crystal formation within the insect body. Damage caused by formation of ice crystals within the cells is generally considered to be lethal, however some exceptions have been reported (see section 1.3.1.). In most insect species, freezing temperatures lead to formation of ice crystals in extracellular fluids. As molecules of water attach to growing ice crystals, the extracellular fluids become more concentrated, which results in osmotic gradient between the unfrozen extracellular fraction and the cytoplasm of the cells. Water is then drawn off the cells resulting in higher concentration of intracellular solutes which may reach toxic levels. In addition, ice crystals alone may cause direct mechanical damage to the cells.

In the case of chill injury, damage is caused by temperature below the threshold for activity but above the temperature of crystallization of body fluids. Chilling injuries can be further divided

into acute (cold shock) and chronic (cumulative). Rapid cooling to relatively low temperatures results in cold shock, which is believed to cause damage predominantly to membranes (Quinn, 1985). The membrane normally exists in a liquid crystalline phase, but when exposed to low temperatures it may transfer into a highly organized gel phase. In the gel phase the membrane lipids exhibit no or minimal mobility, which results in the loss of function of the membrane. Functions of the membrane proteins are also dependent on membrane fluidity. Transition to gel phase may seriously disrupt the membrane transport (Hazel, 1989). At temperatures above optimal level, the membrane may transfer into a hexagonal phase and loose its integrity. Transition to the hexagonal phase may also be caused by low hydration (Kirk et al., 1984). Chronic chilling to relatively mild subzero temperatures negatively influences the function of proteins. Enzymatic activity of proteins decrease with decreasing temperature (Privalov, 1990) and thus ATP production can be disrupted, which results in the collapse of vital processes such as ion transports. Moreover, metabolites may accumulate and reach toxic concentration when metabolic pathways are disrupted.

1.3.3. Ice nucleation, supercooling and water relations in overwintering insects

Homogenous ice nucleation occurs when a sufficient number of hydrogen bonds between molecules of water develop spontaneously at the same location to form a compact initial nucleus. The initial nucleus, when formed, slowly changes its shape and size until it reaches a stage that allows rapid expansion, resulting in crystallization of the entire system (Matsumoto et al., 2002). Homogenous nucleation in a small volume of extremely pure water does not occur until temperatures as low as -39°C. Ice nucleation is a stochastic process dependent on formation of sufficient number of water molecules into ice crystal (Ramløv, 2000; Wilson et al., 2003). The formation of the initial nucleus depends on the number of water molecules available to form it. The probability of ice nucleation thus increases with the volume of water.

Liquid which is cooled below its melting point without a phase transition into solid is called supercooled (Ediger, 2000). Apart from the example of ultra pure water, all other aqueous solutions (including those in living organisms) will undergo heterogeneous nucleation, where a substance other than water serves as the nucleus around which water molecules aggregate. Such substances are called heterogenous nucleators (Lee, 1991; Wilson et al., 2003). Because of the presence of nucleators, larger volumes of tap water usually freeze at temperatures close to 0°C (Doucet et al., 2008). The nucleation activity was described in both organic and inorganic substances (Zachariassen, 1992). Many insect species synthesize hemolymph proteins with their capacity to catalyze ice nucleation at mild subzero temperatures. Ice-nucleating proteins allow insect to control (to a limited extend) the formation of ice crystals within their bodies. Especially insects which tolerate freezing of their body fluids often synthesize these proteins to initiate freezing at relatively high sub-zero temperatures (Storey and Storey, 1988). Freezing at mild subzero temperature allows them to adapt to osmotic pressure caused by the formation of ice as well as direct mechanical action of growing ice crystals (Storey and Storey, 1988; Lee and Costanzo, 1998). In 1970s, a new category of ice nucleators, ice-nucleating microorganisms, was discovered. So-called ice-nucleating bacteria have the capacity to catalyze the formation of ice at temperatures as high as -2°C. Most of these bacteria are epiphytic plant pathogens that facilitate freezing by nucleating water on the plant surface, causing freeze injury.

These bacteria may be responsible for substantial amount of crop losses due to frost (Lee et al., 1996). These bacteria, when ingested by an insect with food, can promote freezing of its body fluids. Some authors reported microorganisms with similar nucleating activity being a part of normal flora of the insect gut (for example: Strong-Gunderson et al., 1990; Lee et al., 1991; Worland and Block, 1999). Many other substances also have ice nucleating activity, but their nature is still not well understood (Lee, 1991). The best ice nucleator available in nature is ice itself. While some species try to avoid contact with external ice, for some other species the contact with ice is crucial to initiate freezing at mild sub-zero temperatures (Lee, 1991). Unlike freeze-tolerant insects, freeze-avoiding insects must avoid formation of ice within their bodies and promote supercooling. Since ice-nucleators of various nature may be present in food, it is essential for freeze-avoiding insects to stop feeding and to evacuate their gut, before the cold season (Sømme 1999).

Removing the ice nucleators is not the only way to regulate ice formation within the insect body. Some insect species synthesize anti-freeze proteins (also called thermal hysteresis proteins) which attach to ice crystals preventing them from further growth (Jorov et al., 2004). The temperature of crystallization is lowered due to the activity of these anti-freeze proteins, but melting temperature remains unchanged. The difference between freezing and melting temperature is called thermal hysteresis and allows us to measure the activity of anti-freeze proteins (Sømme 1999).

When an initial ice crystal reaches a critical mass, surrounding water molecules attach themselves to it very rapidly, which results in explosive freezing in the whole volume of the solution. The heat of crystallization released during freezing allows us to measure the temperature of crystallization, usually referred to as supercooling point. The supercooling point (SCP) is defined as a temperature at which spontaneous freezing occurs (Wilson et al., 2003). In most animals (other than insects), the SCP of body fluids ranges from -0.5°C to -1.7°C (Storey and Storey, 1988). While the SCP refers to the lowest temperature to which an insect may be cooled before it freezes, the supercooling capacity refers to the maintenance of body water in liquid state at temperatures below its equilibrium freezing/melting point (Lee et al., 1996). The ability to manage supercooling is essential for both freeze tolerant and freeze intolerant insect species. Many insects show a seasonal variation in SCP. While in summer the SCP is closer to 0°C, it drops in winter, in some species it even reaches temperatures as low as -60°C (Lee, 1989). Species relying on supercooling strategy lower their SCP during the cold season to prevent freezing of their body fluids. The SCP value is influenced by several factors, such as the portion of osmotically active water and its volume, nucleators of various nature, antifreeze proteins, hydration, or microclimatic conditions of the overwintering site. The SCP value can also be modified by the presence of sugars, polyols and free amino-acids. These substances, called low-molecular mass cryoprotectants (for more information see section 1.3.5.), have an effect on both colligative and non-colligative properties of body fluids and can significantly modify the SCP value and reduce the effect of low temperatures on many biological structures (Lee, 1991; Lee et al., 1996; Zhao, 1997).

The percentage of water by weight in insects usually vary from 65 to 75 % of fresh weight, although the level ranges from only 17% to more than 90% at different life stages of different species (Danks, 2000). Most insect species must face the problem of their water balance during

overwintering. Many insects relying on supercooling strategy overwinter surrounded by ice within their hibernacula. In such a case water molecules tend to evaporate from the body and join the crystals of surrounding ice, which results in desiccation. In order to minimize the loss of water, the cuticular lipid layers become thicker and the composition of cuticular lipids also changes before the cold season (Hegdekar, 1979; Kaneko and Katagiri, 2004). Other mechanical barriers such as cavities build in soil or cocoons also prevent water loss from individuals. Insect cocoons represent a multifunctional barrier, which may protect its occupants from desiccation, penetration of ice crystals and water and also have anti-bacterial and anti-fungal properties (Danks, 2004).

In the case of freeze-tolerant insect species, body fluids become more concentrated as portion of water molecules joins the ice crystals. The unfrozen fraction of body water which is locked in hydration spheres of proteins and many other molecules and particles is called "bound water". In many insects the concentration of solutes increases during overwintering, which results in an increase of volume of bound water at the expense of osmotically active water (free water). Higher amount of bound water (relative to osmotically active water) limits the availability of water molecules for evaporation and ice growth (Block, 2002). As free water molecules join the ice crystals, the osmotic concentration of the unfrozen fraction increases. The vapor pressure of the unfrozen fraction of body water then reaches an equilibrium with the ice. Thus, freeze-tolerant insect should not loose water in favor of external ice.

1.3.4. Cold acclimation

Most diapausing insects require a period of cold acclimation to attain maximum level of cold hardiness. Cold acclimation can be defined as a reversible phenotypic change that occurs in response to declining ambient temperatures and enhances the level of cold hardiness. Cold acclimation is often an integral component of diapause (Salt, 1961; Denlinger, 1991; Šlachta, et al. 2002). Non-diapausing insects can also undergo cold acclimation. The importance of cold acclimation can be illustrated by an example of the fruit fly larva (*Drosophila melanogaster*) - non-diapause stage of a tropical insect species, which becomes cold tolerant during cold acclimation (Overgaard et al., 2008) and can even tolerate partial freezing of its body fluids (Košťál et al., 2012).

Cold acclimation comprises several mechanisms including synthesis and accumulation of so-called low-molecular mass cryoprotectants (for more details see section 1.3.5.) including polyols, sugars and free amino acids (Dubach et al., 1959; Miller & Smith, 1975; Sømme, 1982; Lee, 1991, Sømme, 1999). Other mechanisms include protective changes in the composition of biological membranes. Changes of cell membrane composition represent an important part of the cold acclimation process. Sinensky (1974) proposed the "homeoviscous adaptation theory" (HVA), which postulates changes in membrane composition in order to maintain proper viscosity of the membrane in response to temperature. Because of some discrepancies in observations, McElhaney (1984) introduced a "homeophasic adaptation theory" (HPA), which reflects the need for preservation of the membrane in its functional, liquid crystalline phase. Hazel (1995) broadened the concept to "dynamic phase behavior" (DPB) to stress the dynamism of the phase change. His model assumed a relationship between body temperature

(or ambient temperature) and the temperature of the phase transition of the membrane lipids. In order to prevent transitioning to gel phase during low temperatures, and to keep the membrane functional, changes in membrane composition are made. The effect of low temperature on the membrane is compensated by an increase of unsaturated fatty acids, incorporating shorter chain fatty acids and by increasing the portion of phosphatidylethanolamines relative to phosphatidylcholines. Adjustments of membrane composition in response to temperature changes have been observed in many insect species, for example *Cymbalophora pudica* (Košťál & Šimek, 1998), *Drosophila melanogaster* (Overgaard et al., 2008), *Chymomyza costata* (Košťál et al., 2003), *Pyrrhocoris apterus* (Hodková et al., 1999; Šlachta et al., 2002; Tomčala et al., 2006).

The process of rapid cold hardening, when a short exposure to low temperature extends survival at freezing temperatures, represents a different type of cold acclimation. Rapid cold hardening was first described in the flesh-fly *Sarcophaga crassipalpis* (Chen et al., 1987). The same process was later observed in several other species (Lee et al., 1987; Czajka and Lee, 1990; Qiang et al., 2008). Capacity for rapid cold hardening is a process independent of diapause. It is probably widespread among insects, allowing them to respond quickly to daily temperature oscillations (Lee et al., 1987). Rapid cold hardening can provide a significant advantage especially in spring and autumn when temperature fluctuates and can drop rapidly. The mechanisms responsible for rapid cold hardening are not sufficiently understood. Accumulation of sugars and polyols (Chen et al., 1987, Michaud and Denlinger, 2007), or the synthesis of heat-shock proteins, which represent a universal reaction to rapid temperature changes (Joplin et al., 1990) are considered to be responsible by some authors.

Heat-shock proteins (HSPs) were first discovered in fruit flies exposed to heat (Tissières et al., 1974). HSPs include both inducible and constitutive forms. The constitutive form promotes correct folding of newly synthesized proteins and maintains their function in a normal, unstressed cell. The inducible form serves as molecular chaperone. They bind to partially denatured proteins and mediate either their repair or degradation (Craig et al., 1994). HSPs are not only expressed during a heat shock but also in a response to a variety of other stresses such as cold shock, oxidative and osmotic stress or irradiation (De Maio, 1999). Synthesis of HSPs in response to low temperature was reported in several insect species, for example: *Drosophila sp.* (Goto & Kimura, 1998), *Leptinotarsa decemlineata* (Yocum, 2001) or *Delia antiqua* (Chen et al., 2006). The most complex study has been done on the flesh fly *Sarcophaga crassipalpis* (Joplin et al., 1990; Yocum et al., 1998; Rinehart et al., 2000; Hayward et al., 2005).

1.3.5. Low-molecular mass cryoprotectants

Seasonal changes in cold hardiness of insects are also related to changes in the concentrations of low-molecular mass cryoprotectants in the hemolymph (Sømme, 1999).
The role of low-molecular mass cryoprotectants in overwintering insect was recognized in the middle of 20th century (for example: Salt, 1957; Chino, 1957; Dubach et al., 1959).
Cryoprotectants identified in insects include sugars (trehalose, fructose, glucose etc.), polyols (glycerol, sorbitol, mannitol, ribitol etc.) and free amino acids (proline, alanine etc.) as well (Lee, 1991; Sømme, 1999; Ramløv, 2000). A number of insect species synthesize multiple

cryoprotectants, which may give an advantage - for the concentration of none of the substances reaches a toxic level (Ramløv, 2000).

The cryoprotective role of these substances is based either on colligative or non-colligative properties. High concentrations of cryoprotectants (in order of mol.kg^{-1}) will cause a substantial colligative depression of melting and freezing point of insect hemolymph. In freeze-avoiding insects, the high concentration of cryoprotectants will result in the promotion of supercooling capacity, while in freeze-tolerant insects the cryoprotectants will regulate dehydration of the cells caused by the formation of extracellular ice (Zachariassen, 1985; Lee, 1991; Ramløv, 2000). Non-colligative action of cryoprotectants accumulated at low concentrations (order of tens to hundreds of mmol.kg^{-1}) is based on stabilization and protection of proteins and membranes (Storey and Storey, 1991). The non-colligative effect of solutes is based on mechanism of *preferential hydration* of proteins and membranes or, vice versa, *preferential exclusion* of solvents from the vicinity of proteins and membranes. Solvents and water compete for binding to the protein surface. When solvents are excluded, they allow water molecules to bind preferentially to the protein domain and thus preferentially hydrating it (Timasheff, 2002; Shimizu and Smith, 2004). For example, trehalose is known to be an exceptional stabilizer of proteins; it helps to retain activity of enzymes in solution as well as in their freeze-dried state (Kaushik and Bhat, 2003).

The accumulation of cryoprotectants is influenced by several environmental factors such as temperature, photoperiod and desiccation. The main factor triggering biosynthesis and the accumulation of cryoprotectants is low temperature, usually below 5°C. In many insect species the ability to accumulate cryoprotectants is restricted to diapausing individuals, but in other species, diapause is not a prerequisite for cryoprotectant accumulation (Lee, 1991). Cryoprotectant biosynthesis takes place in fat body tissues and glycogen reserves serve as the main source. Many authors reported conversion of glycogen to cryoprotectants during winter and back to glycogen in spring (for example: Hayakawa & Chino, 1981; Shimada et al., 1984; Rojas et al., 1994; Bemani et al., 2010). The concentration of cryoprotectants in the hemolymph usually shows a seasonal pattern, when the concentration rises from low or even zero in early autumn to its maximum value in mid winter and drops again in early spring (for example: Frankos and Platt, 1976; Li et al., 2000; Atapour and Moharramipour, 2009). The level of cold hardiness (survival at low temperatures and/or SCP) often correlates with the concentration of cryoprotectants (for example: Mansingh and Smallman, 1972; Gehrken, 1984; Chen et al., 1991; Goto et al., 2001; Atapour and Moharramipour, 2009). Some authors, however, reported no correlation between cryoprotectant concentration and cold hardiness, or the seasonal trends of survival/SCP and of cryoprotectants were variously shifted (Pullin et al., 1991; Pullin and Wolda, 1993; Li et al., 2003; Vesala et al., 2012). Pullin (1996) suggested an evolutionary scenario explaining the accumulation of cryoprotectants to be a side-product of metabolic suppression. In this scenario, the diapause-induced metabolic suppression typically results in the accumulation of low concentrations of sugars and polyols, which, later in the course of evolution, may become subject to positive selection for survival in tropical regions (where the compounds might serve as compatible osmolytes) during periods of drought. Further selection of the cryoprotective role of accumulated compatible, osmolytic compounds might have taken place during insect colonization of higher latitudes.

Besides sugars and polyols, some free amino acids seem to have similar cryoprotective role in insects (Lee, 1991; Sømme, 1999; Ramløv, 2000). Košťál et al. (2012) reported a free amino acid proline to have a significant impact on cold hardiness in *Drosophila melanogaster*. Other authors reported an increase of alanin (Li et al., 2001) or alanin and serin (Goto et al., 2001).

1.4. Model species

1.4.1. Codling moth

The codling moth (*Cydia pomonella*) is a major insect pest of apples and some other fruits such as pears, apricots, and walnuts. This species is thought to have originated in Eurasia but later has spread around the world following apple cultivation. It now occurs in most apple production areas in the temperate zone (in both southern and northern hemispheres) but has also been reported in subtropical and tropical countries (Barnes, 1991; Willett et al., 2009). The great economic importance of this pest, and still unresolved difficulties in practical implementation of routine large-scale programs for management of *C. pomonella* in apple orchards (Dorn et al., 1999), are two main drivers of scientific interest into this species.

The codling moth overwinters as a diapausing fifth instar larva in a cocoon spun under the bark of apple trees or in litter near the base of the trees (Miller, 1956; Peterson and Hamner, 1968; Sieber and Benz, 1980). The codling moth has five larval instars regardless of temperature conditions. Optimal temperature for the development of larvae ranges between 28 and 32°C (Williams and McDonald, 1982). In central Europe, the codling moth usually gives rise to one or two generations. When caterpillars reach the stage of fully grown fifth instar larva by approximately half of July, they will continue development and give rise to the summer generation. Most central European populations, however, form only a partial summer generation depending on local weather of that particular year (Miller, 1956) and most caterpillars of the spring generation do not complete their development to adult stage and directly enter diapause (Miller, 1956; Peterson and Hamner, 1968; Sieber and Benz, 1980).

Most of the studies on the codling moth focus on diapause and/or its implications for pest management (for example: Peterson and Hamner, 1968; Riedl and Croft, 1978; Sieber and Benz, 1980; Steinberg et al. 1992a,b). Information about cold hardiness and winter survival are rather sporadic in literature. Several studies report very high mortality (often close to 100%) caused by bird predation of the larvae that overwinter under the bark of apple trees (McLellan, 1958; 1959; LeRoux, 1959; Mailloux and LeRoux, 1960; Solomon et al., 1976; Glen and Milsom, 1978). It is rather surprising, given this high rate of predation, that, according to some authors, overwintering sites under bark are preferred over the litter sites (Gould and Geissler, 1941; McLellan, 1960). Survival rates in litter were not studied in detail. Only two papers report no or very little survival (Solomon, 1976; Glen and Milsom, 1978). Besides predation, the winter cold represents another significant risk for overwintering larvae, but older literature provides only little information about cold hardiness and overwintering in the codling moth larvae (for references see Neven 1999). Only two populations, in the Pacific Northwest in the USA (Neven, 1999) and in the Middle East in Iran (Khani and Moharramipour, 2007; 2010;

Khani et al., 2007) were studied in more detail. Both groups of authors agree on the codling moth being freeze-intolerant and the fact that it does not survive below its SCP, which ranges between -22°C and -24°C. Both groups also reported trehalose to be the major metabolite present in overwintering larvae, but while Khani et al. (2007) found a positive correlation between trehalose concentration and supercooling capacity, no such correlation was reported by Neven (1999). Despite the contributions of these authors, many aspects of cold hardiness and underlying mechanisms are still not completely understood, and no elaborated study is available regarding cold hardiness in central European populations of this species.

1.4.2. Spruce bark beetle

The spruce bark beetle (*Ips typographus*) is the most serious pest of spruce plantations through most of Eurasia (Christiansen and Bakke, 1988; Wermelinger, 2004). Under normal conditions this pest prefers dead or dying trees, but when an outbreak occurs, *I. typographus* changes its behavior dramatically and is able to colonize and kill even healthy growing trees (Christiansen and Bakke, 1988).

In central Europe, when the day-length shortens below 15h, around the middle of August (Schopf, 1985,1989), adult beetles cease their reproduction and enter reproductive diapause. Diapausing beetles overwinter under the bark of spruce trees. Diapause is terminated during December/January (Doležal and Sehnal, 2007) and the beetles then remain in a state of quiescence until temperature begins to rise during the spring, when development and reproduction resume. Several weeks after laying eggs the parental beetles leave their first brood and give rise to a sister brood. The first brood may give rise to a second generation or feed and enter diapause, depending on the photoperiod and temperature. Central European populations of *I. typographus* usually develop two complete generations and several sister broods (Wermelinger, 2004).

Despite the great destructive capability and thus high economic impact of this pest, knowledge of its overwintering is relatively limited. *I. typographus* rely mostly on supercooling strategy (Hansen et al., 1982) but some possibility, at least partial, of freezing tolerance was also reported (Annila, 1969). The only earlier record of polyol analysis, reporting glycerol and glucose to be the major polyols present, is the paper by Hansen et al. (1982) on Estonian population of *I. typographus*. More recently, a paper by Košťál et al. (2007) found glycerol to be only a minor polyol and it described a relatively complex system of cryoprotectants with glucose, trehalose, sorbitol, mannitol and erythritol as major components.

1.4.3. Red firebug

The red firebug (*Pyrrhocoris apterus*) inhabits the western part of the palearctic region but reaches into southern Siberia, Mongolia and China (Stichel, 1959; Puchkov, 1974) as well. In central Europe, *P apterus* clusters, often in large numbers, at the base of linden trees whose seeds are the main component of its diet. Beside linden seeds *P. apterus* also feeds on other plants like mallows (*Malvaceae*) (Tischler, 1959; Socha, 1993) and was reported to be occasionally necrophagous (Southwood and Leston, 1959).

Under natural conditions of central Europe, adults of *P. apterus* enter a facultative reproductive diapause in response to the photoperiod in July and August, and remain in diapause through the end of summer and until early autumn. When temperatures decrease in autumn, the bugs find shelters in the upper litter layer and their diapause is gradually terminated. The bugs then remain in state of low temperature quiescence during winter and resume their activities when the temperature increases in spring (Sláma, 1964; Hodek, 1968, 1983; Hodková, 1999; Košťál & Šimek, 2000; Košťál et al., 2004a,b).

Overwintering *P. apterus* does not tolerate freezing of its body fluids and relies on supercooling strategy with mean SCP of approximately -17°C, with large individual variability ranging from -12°C to -23°C. Diapause is an essential prerequisite for overwintering in P. apterus, because non-diapausing beetles are not capable of cold acclimation nor do they accumulate any polyols (Šlachta et al., 2002). Only diapausing *P. apterus*, when exposed to temperatures below 5°C, can accumulate polyols, which probably function as non-colligative cryoprotectants (Košťál and Šlachta, 2001; Košťál et al., 2001).

1.4.4. Fruit fly

The genus *Drosophila* as well as the whole family *Drosophilidae* is thought to have a tropical origin, but later spread to other climatic zones. Its members now inhabit wide range of environments from the tropics to the edges of tundra. Species of *Drosophila* are a part of saprophytic food chains, because in their immature life stages they depend on organisms causing fermentation (Throckmorton, 1975). The fruit fly *Drosophila melanogaster* has been one of the favorite model organisms, since Thomas Hunt Morgan decided to use it for his research regarding chromosomal theory of inheritance at the beginning of 20th century (Kohler, 1994).

D. melanogaster is relatively easy and cheap to rear in the laboratory. Its life cycle duration is temperature dependent and lasts about 10 days at 25°C (Demerec and Kaufman, 1967). The larval development consists of three stages.

Most of the recent species of *Drosophila* still have tropical and/or subtropical distributions and are chill susceptible (Kohler, 1994). The development of immature stages of *D.melanogaster* halts at temperatures below 10°C (Loeb and Northrop, 1917), some mortality occurs below 6°C (Bliss, 1927), and all developmental stages die when chilled below −5 °C, even if only for a few hours (Czajka and Lee, 1990). Most of the studies on the cold tolerance of *D. melanogaster* focus mainly on cold shock response (Czajka and Lee, 1990; Chen and Walker, 1994; Rajamohan and Sinclair, 2009). Košťál et al. (2012) recently showed, that larvae of *D. melanogaster* can even survive partial freezing of their body fluids, when fed with diet containing proline and acclimated properly.

1.5. Aims of research

Paper I: *Physiological and biochemical analysis of overwintering and cold tolerance in the spruce bark beetle, Ips typographus.*

1) To assess cold tolerance and winter survival in two populations (lowland and highland) of spruce bark beetle (*Ips typographus*) with respect to microhabitat selection.
2) To test physiological limits for survival at low temperatures.
3) To measure physiological parameters such as supercooling capacity, osmolality of body fluids, relative amount of osmotically active water, thermal hysteresis, sugars and polyols, and to examine their relationship to cold tolerance.

Paper II: *Seasonal changes of free amino acids and thermal hysteresis in overwintering heteropteran insect, Pyrrhocoris apterus.*

1) To extend our knowledge of the complexity of physiological adjustments linked to cold tolerance in red firebug (*Pyrrhocoris apterus*).
2) To assess the capacity to stabilize the supercooled state by thermal hysteresis factors.
3) To examine the accumulation of solutes other than polyols, namely free amino acids.

Paper III: *Long-term cold acclimation extends survival time at 0°C and modifies the metabolomic profiles of the larvae of the fruit fly Drosophila melanogaster.*

1) To examine the influence of long-term acclimation on the development of indirect chilling injuries in third-instar larvae of the fruit fly (*Drosophila melanogaster*).
2) To assess the influence of long-term acclimation on ability to survive freezing injury.
3) To examine if long-term acclimation modifies the metabolomic profiles of organic acids, free amino acids, free fatty acids, sugars, and polyols and stimulates restructuring of biological membranes.

Paper IV: *Overwintering strategy and mechanisms of cold tolerance in the codling moth (Cydia pomonella).*

1) To assess cold tolerance and the mortality caused by winter cold in the larvae of Central European population of codling moth (*Cydia pomonella*) on tree trunks and in litter layer.
2) To assess the capacity for survival in supercooled and partially frozen state.
3) To measure changes in water content and energy reserves during overwintering.
4) To measure physiological parameters such as supercooling capacity, thermal hysteresis, osmolality of body fluids, and to perform a detailed metabolomic analysis of organic acids, amino acids, sugars, polyols and free fatty acids end to examine their relationship to cold tolerance.

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2. Results (original publications)

2.1. Paper I

Košťál V., Doležal P., Rozsypal J., Moravcová M., Zahradníčková H., Šimek P. (2011). **Physi-ological and biochemical analysis of overwintering and cold tolerance in the spruce bark beetle, Ips typographus**. Journal of Insect Physiology 57, 1136-1146.

Physiological and biochemical analysis of overwintering and cold tolerance in two Central European populations of the spruce bark beetle, *Ips typographus*

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ARTICLE INFO

ABSTRACT

Overwintering success is one of the key aspects affecting the development and outbreaks of the spruce bark beetle, *Ips typographus* (L.) populations. This paper brings detailed analysis of cold tolerance, and its influence on overwintering success, in two Central European populations of *I. typographus* during two cold seasons. Evidence for a supercooling strategy in overwintering adults is provided. The lower lethal temperature corresponds well to the supercooling point that ranges between −20 and −22 °C during winter months. The supercooled state is stabilized by the absence of internal ice nucleators and by seasonal accumulation of a mixture of sugars and polyols up to the sum concentration of 900 mM. The cryoprotective function of accumulated metabolites is probably based on increasing the osmolality and viscosity of supercooled body fluids and decreasing the relative proportion of water molecules available for lethal formation of ice nuclei. No activity of thermal hysteresis factors (stabilizers of supercooled state) was detected in hemolymph. Lethal times for 50% mortality (Lts50) in the supercooled state at −5, −10 or −15 °C are weeks (autumn, spring) or even months (winter), suggesting relatively little mortality caused by chill injury. Lts50 at −15 °C are significantly shorter in moist (6.9 days) than in dry (>42 days) microenvironment because there is higher probability of external ice nucleation and occurrence of lethal freezing in the moist situation.

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2.2. Paper II

Košťál V., Renault D., Rozsypal J. (2011). **Seasonal changes of free amino acids and thermal hysteresis in overwintering heteropteran insect, Pyrrhocoris apterus.** Comp. Biochem. Physiol. 160A, 245-251.

Seasonal changes of free amino acids and thermal hysteresis in overwintering heteropteran insect, *Pyrrhocoris apterus*

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ARTICLE INFO

ABSTRACT

Overwintering adults of *Pyrrhocoris apterus* do not tolerate freezing of their body fluids and rely on a supercooling strategy and seasonal accumulation of polyols to survive at subzero body temperatures. We sampled the adults monthly in the field during the cold season 2008–2009 and found active thermal hysteresis factors (THFs) in hemolymph of winter-sampled adults. The hysteresis between the equilibrium melting and freezing points ranged from 0.18 °C to 0.30 °C. No signs of THFs activity were found in the autumn- and spring-sampled insects. The total free amino acid pool almost doubled during winter time. The sum concentrations of 27 free amino acids ranged between 35 and 40 mM in whole body water and 40–45 mM in hemolymph during December–February. Two amino acids, Pro and α-Ala most significantly contributed to the seasonal increase, while Gln showed the most dramatic seasonal decrease. Moderate levels of amino acid accumulation in overwintering *P. apterus* suggest that they are by-products of protein degradation and pentose pathway activity during the state of metabolic suppression imposed by diapause and low body temperature. Potential colligative effects of accumulated amino acids, extending the supercooling capacity of overwintering *P. apterus*, are negligible. Non-colligative effects require further study.

2.3. Paper III

Košťál V., Korbelová J., Rozsypal J., Zahradníčková H., Cimlová J., Tomčala A., Šimek P. (2011). **Long-term cold acclimation extends survival time at 0°C and modifies the metabolomic profiles of the larvae of the fruit fly Drosophila melanogaster.** PLoS ONE 6(9): e25025

PLoS one

Long-Term Cold Acclimation Extends Survival Time at 0°C and Modifies the Metabolomic Profiles of the Larvae of the Fruit Fly *Drosophila melanogaster*

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Abstract

Background: *Drosophila melanogaster* is a chill-susceptible insect. Previous studies on this fly focused on *acute* direct chilling injury during cold shock and showed that lower lethal temperature (LLT, approximately $-5°C$) exhibits relatively low plasticity and that acclimations, both rapid cold hardening (RCH) and long-term cold acclimation, shift the LLT by only a few degrees at the maximum.

Principal Findings: We found that long-term cold acclimation considerably improved cold tolerance in fully grown third-instar larvae of *D. melanogaster*. A comparison of the larvae acclimated at constant 25°C with those acclimated at constant 15°C followed by constant 6°C for 2 d (15°C→6°C) showed that long-term cold acclimation extended the lethal time for 50% of the population (Lt_{50}) during exposure to constant 0°C as much as 630-fold (from 0.137 h to 86.658 h). Such marked physiological plasticity in Lt_{50} (in contrast to LLT) suggested that *chronic* indirect chilling injury at 0°C differs from that caused by cold shock. Long-term cold acclimation modified the metabolomic profiles of the larvae. Accumulations of proline (up to 17.7 mM) and trehalose (up to 36.5 mM) were the two most prominent responses. In addition, restructuring of the glycerophospholipid composition of biological membranes was observed. The relative proportion of glycerophosphoethanolamines (especially those with linoleic acid at the *sn*-2 position) increased at the expense of glycerophosphocholines.

Conclusion: Third-instar larvae of *D. melanogaster* improved their cold tolerance in response to long-term cold acclimation and showed metabolic potential for the accumulation of proline and trehalose and for membrane restructuring.

Competing Interests: The authors have declared that no competing interests exist.

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2.4. Paper IV

Rozsypal J, Košťál V, Zahradníčková H and Šimek P (2013) **Overwintering strategy and mechanisms of cold tolerance in the codling moth (*Cydia pomonella*).** PLoS One. (*Article in press*)

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Overwintering strategy and mechanisms of cold tolerance in the codling moth (*Cydia pomonella*).

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Abstract

Background: The codling moth (*Cydia pomonella*) is a major insect pest of apples worldwide. Fully grown last instar larvae overwinter in diapause state. Their overwintering strategies and physiological principles of cold tolerance have been insufficiently studied. No elaborate analysis of overwintering physiology is available for European populations.

Principal findings: We observed that codling moth larvae of a Central European population prefer to overwinter in the microhabitat of litter layer near the base of trees. Reliance on extensive supercooling, or freeze-avoidance, appears as their major strategy for survival of the winter cold. The supercooling point decreases from approximately -15.3°C during summer to -26.3°C during winter. Seasonal extension of supercooling capacity is assisted by partial dehydration, increasing osmolality of body fluids, and the accumulation of a complex mixture of winter specific metabolites. Glycogen and glutamine reserves are depleted, while fructose, alanine and some other sugars, polyols and free amino acids are accumulated during winter. The concentrations of trehalose and proline remain high and relatively constant throughout the season, and may contribute to the stabilization of proteins and membranes at subzero temperatures. In addition to supercooling, overwintering larvae acquire considerable capacity to survive at subzero temperatures, down to -15°C, even in partially frozen state.

Conclusion: Our detailed laboratory analysis of cold tolerance, and whole-winter survival assays in semi-natural conditions, suggest that the average winter cold does not represent a major threat for codling moth populations. More than 83% of larvae survived over winter in the field and pupated in spring irrespective of the overwintering microhabitat (cold-exposed tree trunk or temperature-buffered litter layer).

3. Summary of results and conclusions

Paper I: *Physiological and biochemical analysis of overwintering and cold tolerance in the spruce bark beetle, Ips typographus.*

Cold tolerance and winter survival with respect to microhabitat selection

Our data suggest that low winter temperatures themselves only seldom represent an important mortality factor for Central European populations of I. typographus. Beetles that were sampled during the end of summer and the course of autumn (August–November) 2008 showed relatively low levels of cold tolerance. By the beginning of winter (December), survival at sub-zero temperatures increased considerably in both populations, while survival at an above-zero temperature either remained relatively low (lowland) or increased less conspicuously (highland). The maximum levels of cold tolerance at sub-zero temperatures were achieved during the winter months (December–February, March). A spring loss of cold tolerance was apparent in the population sampled in the lowland, but not in the highland, where March temperatures remained relatively low.

The overwintering success in microhabitats with high moisture (litter and moss, under bark of fallen trees) may be negatively influenced by relatively high risks of lethal nucleation by external ice. The survival at longer exposure times was markedly lower in moist than in dry conditions, which provides an additional explanation why most beetles prefer overwintering sites on standing trees over litter.

Physiological and biochemical parameters of cold tolerance

Lower lethal temperature corresponds to the beetle supercooling point which ranges between -20 and -22°C during winter. Beetles collected during the end of summer (August) displayed relatively low supercooling capacity (lowland, average SCP = -11.7 °C; highland, average SCP = -12.1 °C). SCPs then gradually decreased during autumn and reached a seasonal minimum during winter (ranging between -20 and -22 °C during December–March in both populations). Spring (April) beetles lost their high supercooling capacity (lowland, average SCP = -6.6 °C; highland, average SCP = -8.6 °C). Osmolality of hemolymph followed a seasonal pattern that was almost reciprocal to SCP. Average values of 335 and 527 mOsm kg^{-1} were measured in lowland and highland, respectively, during the end of summer. Winter maxima rose to 1359 and 1582 mOsm kg^{-1} in lowland and highland, respectively. Spring levels (April) were again low: 454 and 401 mOsm kg^{-1} in lowland and highland, respectively. The osmolality of hemolymph tightly correlated with the sum concentration of all sugars and polyols.

Accumulation of cryoprotectants

The supercooled state appears to be well stabilized by the absence of internal nucleators and by high concentrations of sugars and polyols which increase the osmolality and viscosity of body fluids on the one hand and decrease the relative proportion of osmotically active water molecules on the other. The levels of trehalose were stable during early autumn, increased steeply starting in September (highland) or October (lowland), reached maximum levels during winter months and, finally, decreased during spring (a decrease was observed only in the sample taken in the lowland). The concentrations of glucose followed a similar seasonal pattern as trehalose. Sorbitol, mannitol, erythritol, threitol, fructose (and glycerol during 2009–2010) occurred at very low or undetectable levels during autumn, rapidly accumulated during early

winter (December in highland, January in lowland), and were maintained at high levels during winter.

Thermal hysteresis factors and ratio of osmotically active and inactive water
No signs of thermal hysteresis factors were found in hemolymph of overwintering beetles. The fresh mass and hydration of overwintering beetles remained essentially constant. We observed significant seasonal changes in the relative proportion of osmotically active (OA) vs. inactive (OI) water. Beetles collected during August 2010 showed a relatively high proportion of OA water (74 %), which then decreased to 55 - 60 % during the winter months.

Paper II: *Seasonal changes of free amino acids and thermal hysteresis in overwintering heteropteran insect, Pyrrhocoris apterus.*

Stabilization of supercooled state by thermal hysteresis factors
We detected active thermal hysteresis factors (THFs) to be present consistently in all winter-collected adults of P. apterus, while no signs of THFs activity were found in the autumn- and spring-collected insects. The average levels of THFs activity was relatively low, ranging from 0.18°C to 0.30°C, which is not sufficient to significantly extend the supercooling capacity of P. apterus.

Accumulation of free amino acids
We found that free amino acids accumulate in overwintering adults of P. apterus, however, their contribution to the observed change in hemolymph osmolality is relatively small, up to 20 mOsm. The total free amino acid pool almost doubled during winter time compared to early September in both hemolymph and whole body samples. Three amino acids, Proline, α-Alanine and Glutamine, represented between 48 and 67 % of the total amino acid pool. The levels of Proline and α-Alanine increased with the progression of cold season, peaked during winter, and decreased during early March. Concentration of Glutamine showed a reciprocal trend with a broad minimum during winter.

Paper III: *Long-term cold acclimation extends survival time at 0•C and modifies the metabolomic profiles of the larvae of the fruit fly Drosophila melanogaster.*

Effect of long-term acclimation on survival at low temperatures
We observed a clear effect of relatively low rearing temperature of 15°C on subsequent survival at low temperatures in 3rd instar larvae. Survival of the 15°C acclimated larvae further improved on exposure to 6°C for 2 days. The larvae did not show survival ability in conditions favorable for external ice inoculation and partial freezing of their body fluids. None of the larvae survived cooling to -5°C when freezing of the surrounding diet was stimulated by adding a small ice crystal. In contrast, relatively high proportions of the larvae survived cooling to -5°C under the supercooling conditions. However, acclimation at 15°C or 15°C → 6°C was a prerequisite for their survival in supercooled state.

Effect of long-term acclimation on metabolomic profiles and restructuring of membranes
Almost two-thirds of the 39 major metabolites identified in this study exhibited statistically significant concentration changes in response to long-term cold acclimation. Most of the

changes, however, were relatively small and rarely reached a several-fold magnitude. Two compounds, trehalose and proline, were present in relatively high amounts (>10mM) and showed a positive association with increasing cold acclimation.

Long-term cold acclimation stimulated small but statistically significant changes in the lipid composition of biological membranes in D. melanogaster larvae. The relative proportion of unsaturated FAs and the length of FA chains did not change significantly with cold acclimation. The relative proportion of GPEtns was significantly lower in the 25°C- and 15°C-acclimated larvae than in the 15 °C → 6°C-acclimated larvae.

In both the larvae and the adults of D. melanogaster, the most prominent change related to cold acclimation was the increase in the relative proportion of GPEtns with linoleic acid (FA 18:2) esterified at the sn-2 position of glycerol.

Paper IV: *Overwintering strategy and mechanisms of cold tolerance in the codling moth (Cydia pomonella).*

Overwintering sites, cold tolerance and the mortality caused by winter cold

Our data suggest that low temperatures do not represent a major threat for codling moth populations, when considering the conditions of an average winter. The cold tolerance in field collected larvae increased gradually with seasonal time and remained at high level until spring. The larvae survived equally well in the litter layer (86.1% survival) and on tree trunks (83.6% survival). When considering the conditions of an extremely cold winter, the survival rate would most likely be zero, provided Codling moth larvae overwinter in the exposed microhabitats of tree trunks. We believe, however, that larger parts of Central European populations prefer overwintering sites in the buffered microhabitat of the litter layer.

Survival in supercooled and partially frozen state

Our study confirms supercooling as the main strategy of cold tolerance but also shows that the overwintering larvae of *C. pomonella* possess a good physiological capacity for freeze-tolerance. The July-collected non-diapause larvae displayed relatively low capacity to tolerate subzero temperatures. They also did not tolerate freezing of their body fluids. In diapausing larvae, the cold tolerance increased gradually with seasonal time and reached a broad plateau between November and April. The capacity to tolerate freezing was first observed in November-collected larvae and, later, it stayed very high, ranging between 75-100%, until spring.

Water content and energy reserves during overwintering

We found that larvae of codling moth gradually lose water during overwintering. This partial dehydration contributes to the increase of body fluids' osmolality that, in turn, correlates with the decrease of SCP. During the almost 6-month-long overwintering period from November to April, the larvae displayed considerable losses of fresh mass (average loss of 43.0% of initial FM), dry mass (43.8%), total lipids (46.0%) and water (56.8%). Despite these losses in absolute units, the relative contents of water and total lipids remained almost unchanged: water, 61.9% in Nov *vs.* 61.7% in Apr; total lipids, 11.7% in Nov *vs.* 11.1% in Apr.

Whole-body glycogen content was approximately half in July-collected non-diapause caterpillars when compared to September-collected diapausing caterpillars. High levels of

glycogen were maintained during the whole autumn. Practically all glycogen deposits were depleted between November and January, and partially re-accumulated during the spring.

Physiological and biochemical parameters of cold tolerance

The osmolality of hemolymph was relatively low (252 mosmol kg^{-1}) in July-collected non-diapause larvae. In diapausing larvae, the osmolality gradually increased during autumn from 370 mosmol kg^{-1} in September to a broad maximum of 667 - 665 mosmol kg^{-1} in January - March, respectively. The April-collected larvae exhibited a slight decrease of osmolality to 414 mosmol kg^{-1}. Supercooling capacity was relatively low in the July-collected non-diapause larvae. The SCP gradually decreased with seasonal time, reached a minimum during March (-26.3°C), and also remained very low in the April-collected caterpillars. The correlation between hemolymph osmolality and whole body supercooling point (SCP) was close to statistical significance.

No thermal hysteresis was detected in the non-diapause larvae, and extremely low (ranging between 0.07°C to 0.11°C) in hemolymph samples of winter collected larvae.

Accumulation of several metabolites, dominated by fructose and alanine, represents an additional source of increasing osmolality/decreasing SCP in overwintering larvae of codling moth. The concentration of trehalose was relatively high and more or less stable. The levels of fructose, glucose, sorbitol and mannitol, appeared in high concentrations between November and January but were almost completely cleared between March and April. Total pool of free amino acids increased during winter and alanine contributed most to the winter peak. Proline was the second most abundant amino acid and its seasonal pattern resembled that of trehalose - relatively stable.

4. Curriculum vitae

Jan Rozsypal (*1st August 1982, Brno)

Education

Since 2009: University of South Bohemia, Faculty of Science, PhD. studies, Physiology and developmental biology; PhD. thesis: Insect overwintering: physiological and biochemical adaptations to low temperatures [in English].

2006 – 2008: University of South Bohemia, Faculty of Science, MSc. Studies, Zoology; Msc. thesis: An experimental research on predator recognition in birds [in Czech].

2002 – 2006: University of South Bohemia, Faculty of Science, B.Sc. studies, Biology; B.Sc. thesis: How do birds recognize their predators and competitors [in Czech].

Teaching activities

2009 – 2010: Agricultural zoology (practice)

2010 – 2011: Physiology of animals (practice)

2012: Field course for 1st grade students

2009 – 2012: Methodological guidance of undergraduate students

Conferences

Rozsypal J, Poláková S and Fuchs R (2008): Lze rozpoznávání a hodnocení predátorů studovat v klecových experimentech? (*Testing predator recognition and discrimination in laboratory experiments*). Zoologicke Dny 2008, Czech Republic (poster).

Rozsypal J (2010) Physiology of diapause and cold tolerance in prepupae of codling moth, *Cydia pomonella.* ICIRD 2010, Czech Republic (poster).

Papers

Košťál V, Doležal P, **Rozsypal J**, Moravcová M, Zahradníčková H and Šimek P. (2011) Physiological and biochemical analysis of overwintering and cold tolerance in two Central European populations of the spruce bark beetle, Ips typographus. *Journal of Insect Physiology* **57**: 1136-1146.

Košťál V, Renault D, **Rozsypal J** (2011) Seasonal changes of free amino acids and thermal hysteresis in overwintering heteropteran insect, Pyrrhocoris apterus. *Comparative Biochemistry* and *Physiology A* **160**: 245-251.

Košťál V, Korbelová J, **Rozsypal J**, Zahradníčková H, Cimlová J, Tomčala A and Šimek P (2011) Long-term cold acclimation extends survival time at 0°C and modifies the metabolomic profiles of the larvae of the fruit fly Drosophila melanogaster. *PLoS ONE* **6**: e25025.

Rozsypal J, Košťál V, Zahradníčková H and Šimek P (2013) Overwintering strategy and mechanisms of cold tolerance in the codling moth (*Cydia pomonella*). *PLoS ONE*. (Article in press)

Košťál V, **Rozsypal J**, Pech P, Zahradníčková H and Šimek P (2013) Physiological and biochemical responses to cold and drought in the rock-dwelling pulmonate snail, Chondrina avenacea. *Journal of Comparative Physiology B*. (Article in press)

Internships

2011: Kochi University, Graduate school for integrated arts and sciences, Laboratory of environmental physiology**.** 2-5-1 Akebono-cho*,* Kochi 780-8520, Japan

Other activities

2011: Presentation of Laboratory of Insect Diapause, Institute of Entomology, BCAS CR at Kyoto University, Japan