Faculty of Science
University of South Bohemia
České Budějovice

Ph.D. Thesis

# Physiological and molecular adaptations during diapause development and overwintering in a heteropteran bug, *Pyrrhocoris apterus*

Michaela Borovanská

České Budějovice 2009

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České Budějovice 2009

**Borovanská, M**., 2009: Physiological and molecular adaptations during diapause development and overwintering in a heteropteran bug, *Pyrrhocoris apterus*. Ph.D. Thesis, in English. 120 p., Faculty of Science, University of South Bohemia, České Budějovice, Czech Republic.

## Annotation:

In this thesis I present complex experimental data on the physiological and molecular adaptations during diapause development and overwintering in a linden bug, *Pyrrhocoris apterus* (Heteroptera, Pyrrhocoridae). I focus on adjustments of the enzymatic complement, which is involved in the biosynthesis of cryoprotectants, and heat shock proteins, which are expressed in response to temperature stress.

## Financial support:

This work was supported by the Grant Agency of the Czech Republic (Grant No. 206/03/0099), Czech Science Foundation (Grant No. 206/07/0269; 206/05/2085) and by the Grant Agency of University of South Bohemia (GAJU 37/2006/P-BF).

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## Acknowledgement:

I would like to thank to my supervisor, Vladimír Košťál, for guiding me with friendly and patient attitudes during invaluable productive eight years spent in his laboratory, for his help with our experiments and constructive remarks in preparation of the manuscripts. I am also grateful to the laboratory team (Irena Vacková and all Czech and foreign students spending some time in our lab) for its kind atmosphere and assistance. My special thanks go to my family, husband and friends for their love, patience and neverending support.

## Co-authors agreements:

We declare that Michaela Tollarová (Borovanská) contributed to these studies in a great extent. She significantly participated in design of the experiments, she prepared reagents and materials and she accomplished most of the experimental work. Finally she partly analyzed the data.

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Vladimír Košťál

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Jan Šula

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Aleš Tomčala

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David Doležel

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Insects belong to the most successful groups of organisms that have evolved numerous adaptations to seasonally oscillating environments. Decreasing temperatures in winter or dry seasons in tropics represent the most frequent conditions, which are incompatible with insect development and active life.

This chapter should introduce the reader to our current knowledge on the way how insects have adapted to seasonally recurring unfavourable conditions through the evolution of diapause and cold hardiness. A general perspective will be outlined and some well-documented examples will be presented. Physiological, biochemical and molecular mechanisms to cope with low temperatures are in the centre of my interest. In my Thesis, I focused on adjustments of the enzymatic complement, which is involved in the biosynthesis of cryoprotectants, and on heat shock proteins, which are expressed in response to temperature stress. The overwintering linden bug, *Pyrrhocoris apterus* (Heteroptera, Pyrrhocoridae), served as a model species for my studies.

# Diapause

In the temperate zone habitats, growth and reproduction of ectotherms are restricted to a warm part of the year (summer), because low winter temperatures limit availability of food resources and directly decrease rates of biochemical reactions, metabolism, and life functions. In addition, winter season brings other environmental stresses such as risk of chilling and freezing injuries, risk of desiccation, attack by pathogens and predators. In response to seasonally predictable occurence of unfavourable conditions, a form of dormancy – diapause, which is analogous to hibernation in mammals, has evolved in many insect species (Andrewartha, 1952; Lees, 1955; Danilevski, 1961; Hodek, 1983; 1996; 2002; Tauber et al., 1986; Danks, 1987).

Diapause represents an alternative developmental pathway characterized by a complete halt (or considerable slowing) of development, suppression of metabolism, and overall deep change of phenotype starting from unique gene expression and ending with altered physiology, morphology and behaviour (Denlinger, 1985; 2000; 2002). Diapause is considered as a dynamic process consisting of several successive phases, rather than as a static developmental arrest (reviewed in Košťál, 2006). The terminological system suggested by Košťál (2006) will be used in this Thesis. Three main phases of ontogeny will be distinguished: pre-diapause development, diapause and post-diapause development; within the phase of diapause itself, the phases of initiation, maintenance and termination are clearly separable (at least in *P. apterus*) by using various physiological and molecular tools (Košťál et al., 2008).

Diapause occurs at a fixed, species-specific ontogenetic stage. For example, the silkmoth, *Bombyx mori* diapauses as early embryo (Yamashita, 1996), the European corn borer, *Ostrinia nubialis* enters diapause in its larval stage (Beck, 1989), the flesh fly, *Sarcophaga crassipalpis* in pupal stage (Denlinger, 1971), and the Colorado

potato beetle, *Leptinotarsa decemlineata* in adult stage (Lefevere & DeKort, 1989). In some (relatively rare) cases, diapause is a fixed part of ontogenetic programme, which implies that the insect will always enter diapause regardless of the environmental conditions (obligatory diapause). In most cases, however, the induction of diapause is determined by specific environmental stimuli (facultative diapause).

The most common signal that regulates entry into diapause is short daylength during the late summer or early autumn. Photoperiodic signal (critical photoperiod) enters the brain either through the eyes or via extraretinal receptors (Numata et al., 1997), it is then processed by unknown pathways in central nervous system (Stehlík et al., 2008), and transduced into a deep change of hormonal milieu, which causes the characteristic changes in physiology, morphology and behaviour of the insect (Denlinger, 1985; 2000; 2002). Although the daylength is a dominant environmental cue, other factors such as temperature, food quality and quantity, population density, and ecological interactions with other species may contribute to the incidence of diapause (Košťál, 2006).

Many studies have been conducted over the past years on hormonal regulation of diapause (for reviews, see: Denlinger, 1985; 2000; 2002). Precise functioning of hormonal systems is dependent on the species and developmental stage in which diapause occurs. Considering that insect hormones affect enormously wide spectrum of physiological processes, while literature usually mentions very specific roles in specific species, it is not simple to create a complex generalization on endocrine regulation of diapause. Two major families of hormones are involved in the regulation of diapause: juvenile hormones (produced in neurohemal organ corpora allata) and ecdysteroids (secreted by prothoracic gland). Their synthesis and secretion are regulated by direct nervous connections from the central brain and by neuropeptides, also secreted by specialized neurons of the central brain (Hodková, 1976; 1999; Hodková et al., 2001). Specific neuropeptide regulator of embryonic diapause, diapause hormone, was revealed and characterized in *B. mori* (Yamashita, 1996; Zhang et al., 2004). Diapause hormone, which is derived from pheromone biosythesis activating neuropeptide (PBAN), recently appeared to be an important regulator of development and reproduction in other moth species, e.g. *Manduca sexta* (Xu & Denlinger, 2004), *Heliothis virescens* (Xu & Denlinger, 2003) or *Helicoverpa armigera* (Wei et al., 2005).

Prior to entering diapause, the insects accumulate nutrient reserves during preparation or initiation phases (Košťál, 2006; Hahn & Denlinger, 2007). The fat body is a primary site of glycogen and triacylglycerol reserves synthesis, storage and catabolism (Hahn & Denlinger, 2007). Free amino acids and hexameric proteins are stored in haemolymph (Šula et al., 1995). In general, the energy reserves are continuously depleted during diapause progression, especially in the non-feeding diapause stages (embryo, immobile prepupa, pupa). The patterns of reserve utilisation, however, differ in individual species (Chippendale, 1973; Adedokun & Denlinger, 1985; Lefevere & DeKort, 1989).

The rate of metabolic supression depends on insect species and its stage of diapause, but in general, it is important to shut off or downregulate the rates of

energetically expensive and temperature sensitive physiological systems such as: cell proliferation, differentiation and morphogenesis; intense locomotion or flight; feeding, digestion and metabolism; maturation of gonads, mating and reproduction (Denlinger, 2002; Storey & Storey, 2004). The pathways of basic intermediary metabolism and those ensuring cellular homeostasis are maintained. Specific metabolic processes may be even up-regulated during diapause (for instance those leading to cryoprotectant accumulation – will be discussed later). As a rule of thumb, the overall ATP-producing and ATP-consuming processes remain balanced (Storey & Storey, 2004). Metabolism can also exhibit regular cycles of activity during diapause (Denlinger et al., 1972).

Techniques of molecular genetics represent the most progressive tool added to the insect physiologist´s armoury in last few years. We are now in a position to start connecting the emerging knowledge on molecular mechanisms with the well-characterised physiological responses and thus contribute to the opening of „the regulatory Black Box“ between the perception of environmental signals and the expression of diapause phenotype. Molecular information on diapause, however, is currently limited to a few insect species. Even though diapause and overwintering expectedly result in silencing or down-regulation of many genes, a small subset of genes is upregulated or uniquely expressed during diapause (Denlinger et al., 1995; Flannagan et al., 1998). The differentialy regulated genes were divided into several categories: cell cycle regulators, signal transduction systems, molecular transporters, transcription factors, stress response, food utilization, metabolic function. Expression of some upregulated genes persists throughout the whole unfavourable period; others are upregulated only in early or late stages (Rinehart et al., 2000; Denlinger, 2002; Robich et al., 2007).

One characteristic feature of developmental arrest is a cell cycle arrest. Cell division cycle is halted in G0/G1 phase in diapausing pupae of *S. crassipalpis* (Tammariello & Denlinger, 1998) or in G2 phase in diapausing embryo of *B. mori* (Nakagaki et al., 1991). Expression of one of the important cell-cycle regulators, Proliferating Cell Nuclear Antigen (PCNA), is strongly suppressed in diapausing *S. crassipalpis* (Tammariello & Denlinger, 1998; Hayward et al., 2005). Recently, the genes of MAP kinase family, important components of signal transduction cascades, have been cloned and studied during embryonal diapause in *B. mori* (Fujiwara et al., 2006). Study of insulin signaling pathways and FOXO gene (forkhead transcription factor) expression during overwintering diapause of a mosquito, *Culex pipiens* represents another step forward in understanding of molecular mechanisms mediating diapause response (Sim & Denlinger, 2008).

Many other studies have been done on gene products expressed exclusively either in diapause or during active development. For example, the expression of actin gene in various tissues were studied in *Lymantria dispar* (Lee et al., 1998) or *C. pipiens* (Kim et al., 2006). So called short neuropeptides (sNPF), characterized in *L. decemlineata*, are present only in non-diapause stages (Huybrechts et al., 2004). In contrast, diapausing pupae of this species specifically express the diapause protein 1 (de Kort & Koopmanschap, 1994), juvenile hormone estherases (Vermunt et al., 1999) and some

other diapause associated transcripts (Ld DAT 1, 2, 3) (Yocum, 2003). Increased abundance of haemolymph proteins, generally referred to as hexameric storage proteins, are characteristic in diapause. Such proteins have been well documented in *D. grandiosella* (Brown & Chippendale, 1978), *Cydia pomonela* (Brown, 1980), *Pectinophora gossypiella* (Salama & Miller, 1992), *P. apterus* (Šula et al., 1995), *L. decemlineata* (Koopmanschap et al., 1995), *Choristoneura fumiferana* (Palli et al., 1998). These proteins do not appear to be markers of diapause or overwintering programme; they serve as an important amino acid source for metabolism and tissue development that occurs immediately after the termination of developmental arrest.

Up- or down-regulation of genes coding for various metabolic enzymes have been studied rather randomly; first molecular studies have appeared in last few years. For instance, Robich and Denlinger (2005) observed changes in the expression of genes coding for trypsin, chymotrypsin-like serin protease (enzymes needed to digest a blood meal) and fatty acid synthase (key enzyme associated with the accumulation of lipid reserves) in overwintering females of the mosquito *C. pipiens*. Genes encoding two blood-digestive enzymes are down-regulated in early diapause, and a gene encoding an enzyme involved in lipid sequestration is concurrently highly up-regulated in diapause-destined females. This molecular evidence demonstrates a metabolic switch from non-diapause blood feeding to sugar feeding and lipid sequestration in diapause.

## Diapause in *P. apterus*

The red firebug, *Pyrrhocoris apterus*, is a model species used for studies presented in this Thesis. In natural conditions of South Bohemia, the adults of brachypterous wing-morph enter facultative reproductive diapause in response to the short days of July and August, and maintain diapause during the warm end of summer and the beginning of autumn. During decreasing temperatures in late autumn, they find shelters in the upper litter layer and the diapause is gradually terminated. During cold winter months adults persist in a state of low temperature quiescence until the vernal rise of temperatures resume their locomotion, feeding and reproduction activities (Sláma, 1964; Hodek, 1968, 1983; Hodková, 1999; Košťál & Šimek, 2000; Košťál et al., 2004a,b).

The critical day-length of ca. 16 h 30 min serves as a pivotal signal inducing entry into diapause in the Czech populations of *P. apterus* is (Hodek, 1968). The question whether the products of various clock genes are or are not involved in photoperiodic regulation of diapause is currently subjected to detailed investigation (Hodková et al., 2003; Syrová et al., 2003; Doležel et al., 2007). Principles of the transduction of photoperiodic signal to neuroendocrinne level were described by Hodková et al. (1999, 2001). By using surgical interventions to the neuroendocrine complex, they identified neuronal and humoral regulatory pathways leading to onset of diapause development. Alteration in hormonal signalling pathways in turn influence numerous

physiological targets including reproduction activity. Diapausing females do not mate and do not develop and lay eggs. The duration of pre-ovipostion period (POP) after the transfer of diapausing females to permissive conditions (high temperature of 25°C and long day of 18 h L : 6 h D) may serve as a good descriptor of the gradual changes in the intensity of diapause (*i.e.* the progression of diapause development). It has been shown in both field-collected and laboratory-reared bugs that the POP gradually shortens with the progression of diapause development (Hodek, 1971; Socha & Šula, 1992; Košťál et al., 2008). Other physiological changes that are linked to initiation phase were observed. For instance: the respiration rate (Sláma, 1964), the feeding and drinking rates (Socha et al., 1997), and locomotor activity (Hodková, 2003), all decreased significantly; transient changes in the activities of several digestive enzymes were detected (Socha et al., 1997); nutrient reserves rapidly accumulated in the fat body and muscles (Šula et al, 1997; Košťál et al., 2004a); and the concentrations of storage hexamer proteins increased in the haemolymph (Šula et al., 1998). In this Thesis, we add detailed observations of the diapause-related changes in relative abundance of mRNA transcripts of eight different genes coding for proteins implemented in energy metabolism, cryoprotectant biosynthesis, biological clocks, and hormonal receptors (Košťál et al., 2008).

## Cold acclimation and cold hardiness

Temperature affects every aspect of an ectotherm´s biological function. Rates of biochemical reactions and metabolism generally increase with increasing body temperature (of course, only up to some point of temperature optimum, where they turn to decrease). Development and other complex life processes are also temperature dependent. Insects' ability to regulate body temperature is only limited. Mostly, thermoregulation is achieved by using behavioral strategies such as basking/sheltering or muscle vibrations. There are insect species which can survive either extreme subzero temperatures of -50°C while some other species are adapted to survive at very high temperatures over 50°C. Adaptations and acclimatory changes in response to low temperature are in focus of this Thesis.

Any temperature below the thresholds for activity, growth and development may be considered as low. In a more strict sense, however, only *the temperatures which negatively influence the insect physiology and cause injury are considered as low temperatures* (Lee & Denlinger, 1991; Nedvěd, 1998). The exact range of low temperatures is species-specific and depends on the insects' eco-physiological state. Therefore many tropical and warm-acclimated temperate insect species may die at temperatures highly above 0°C, for example at 15-20°C. But most of the studies were focused on the effects of subzero temperatures (< 0°C).

Ectotherms (including insects) have evolved a diversity of behavioural, morphological and physiological adaptations for survival in cold during their evolution. The sum of such adaptations is collectively understood as cold hardiness.

Cold hardiness can be simply defined as the ability of an organism to survive at low temperatures (Salt, 1961; Danks, 1978; Zachariassen, 1985; Bale, 1989; Michaud & Denlinger, 2004). The fundamental evolutionary *adaptation* of cold hardy organisms is their capability of *acclimatization* and, especially, *cold acclimation.* Cold acclimation, which represents typical part of complex seasonal acclimatization process, is characterized as a transient phenotypic change (plasticity) that occurs in response to declining ambient temperatures. Cold acclimation is often an integral component of diapause (Lee & Denlinger, 1991; Šlachta et al., 2002). In some species, however, diapause and cold hardiness may occur independently (Lee & Denlinger, 1991).

Two broad categories of insect overwintering strategies are recognized: „freeze-avoidance“ and „freeze-tolerance“ (Lee & Denlinger, 1991; Sinclair et al., 2003). Freeze-avoiding insects cannot survive ice formation in their body fluids and often die well above the temperature of crystallization of their body fluids, also known as a supercooling point (Bale, 1993; Renault et al., 2002). Freeze-tolerant species survive partial freezing of their body fluids, provided this is restricted to extracellular compartments (see below for exceptions). Although the two strategies are fundamentally different, they share several similarities, and certain species may even switch from one strategy to the other from one year to the next (Horwath & Duman 1984).

Precise physiological principles of cold injury are still poorly understood. The main reason for such uncertainty is that the changes in body temperature target all levels of biological organization. Enormous number of processes and structures change with increasing/decreasing body temperature. Hence, it is not easy to decide on importance and causality of individual changes. Cold injury can be divided into two main categories: freeze-injury and chill-injury (Storey & Storey, 1988). In the first case, damage occurs as a result of ice crystal formation. In the second case, damage is caused by temperatures that are below the threshold for activity but above the temperature of crystallization of body water (i.e. real freezing point or supercooling point, SCP).

Intracellular freezing is generally considered to be lethal (Asahina, 1969; Zachariassen, 1985). However, at least one nematode, *Panagrolaimus davidi* breaks the rule and shows what was long thought as impossible - it survives extensive intracellular freezing (Wharton & Ferns, 1995). In addition, Salt (1962) and Davis & Lee (2001) reported that the isolated fat body cells of dipausing larvae of *Eurosta solidaginis* also can survive intracellular freezing. In the great majority of freeze-tolerant insects studied so far, freezing temperatures lead to ice crystal formation outside the cells. It results in an osmotic gradient between the unfrozen extracellular fraction and the interior of the cell. The osmotic disbalance across the membrane forces the water to leave the cell, dehydrating it and increasing the concentration of cytoplasmic solutes. High concentrations of intracellular solutes cause dramatic changes in pH, fluid viscosity, protein structure and enzyme functioning, which can be highly damaging to cells and tissues of unadapted/unacclimated insect.

Furthermore, direct mechanical action of ice crystals on cytoskeleton and cellular membranes is another important source of freeze-injury.

The physiological nature of chill-injury is much less understood. At a cellular level, it may be caused by: (i) loss of membrane barrier function and leakage of solutes, (ii) changes in protein conformation, protein instability, depolymerization and/or denaturation; and (iii) overall metabolic disorder.

Ad (i): The physical properties of lipidic bilayers, i.e. the *phase* state and the *fluidity*, are acutely sensitive to temperature. Three basic *phase* states exist: highly ordered bilayer formed by lipids in a lamellar gel phase ($L_{ß}$); fluid bilayer, liquid crystalline phase ($L_{\alpha}$) and; nonbilayer, reversed hexagonal phase ($H_{II}$) (Chapman, 1975). Membrane fluidity is affected by various factors such as chemical composition, degree of hydration, pressure and temperature. Generally, at low temperatures, the membrane lipids become organized more rigidly in a gel phase. They are locked in place and exhibit neither flip-flop nor lateral mobility. As the temperature increases, it reaches a specific phase transition temperature ($T_m$), a certain melting temperature at which the membrane changes from solid phase to liquid phase, where the bilayer thickness is reduced, its volume increases and rapid translational movements of individual lipid molecules are allowed. Further increases of temperature may cause the transition into a nonbilayer, hexagonal phase at a specific temperature ($T_h$), where the bilayer loses its integrity. Transition to the $H_{II}$ phase is favored by low hydration rates (Kirk et al., 1984). Those insects which overwinter in a frozen or dehydrated state may need specific adaptations to avoid transition of their membranes into the hexagonal phase (Pruitt & Lu, 2008). Permeability and activities of membrane bound enzymes, are also directly dependent on membrane state of fluidity. These functions tend to decrease gradually with decreasing temperature even when the "functional" fluid phase is maintained (Cossins & Macdonald, 1989; Hazel, 1989). After the transition from fluid to gel phase the activities of membrane bound enzyme and transport systems are drastically reduced (Hazel, 1989). Moreover, as the membranes are composed of many diverse lipid species (Dowhan, 1997), the phase transitions may span relatively broad temperature ranges. Thus, the gel and fluid membrane areas may coexist at certain temperatures. It can result in rapid loss of barrier function. Formation of a gel phase is thus believed to impend the cell functionality and survival. Similarly, unregulated transitions in the $H_{II}$ phase are considered incompatible with life processes.

Ad (ii): Protein depolymeration usually occurs at temperatures as high as 0°C and lower temperatures may result in irreversible protein denaturation (Privalov, 1990). At quarternary structure level, protein subunits are dissociated with decreasing temperature. Hereafter, all proteins lose their optimal noncovalent interactions with surrounding water and ion molecules and with the other proteins and their proper functioning is limited. For example, enzymes or protein transporters lose their activity, because substrates or transported molecules, respectively, can no longer bind to the active sites, which are not correctly positioned.

Ad (iii): Metabolism critically depends on concerted and highly regulated activities of many enzymes. With decreasing temperature, the enzymatic activities of proteins

generally decrease. The problems may arise when different enzymes have different temperature requirements and different responses to temperature change. Energetic resources may become depleted as the ATP production is disrupted. Thus, key ATP-dependent processes, such as ion pumping, may collapse. In addition, metabolic intermediates may be accumulated and reach toxic concentrations. In brief, metabolic pathways are in disbalance at sub-optimal temperatures.

Most of the overwintering insects must face a problem with their water balance. Many supercooled insects hibernate surrounded by ice crystals and water molecules tend to evaporate from their body and join the surrounding ice. In order to avoid or minimize such loss of water (or, vice versa, to prevent the penetration of ice crystals into the body), cuticular lipid layers become thicker and the composition of cuticular lipids may change with preparation for overwintering (Kaneko & Katagiri, 2004). In frozen insects, the vapour pressure of the unfrozen fraction of the body fluids is in equilibrium with ice, hence, freeze-tolerant insects should not lose water to surrounding ice. So called "bound water" is associated with hydration spheres of proteins, glycogen, aminoacids, polyols and ions. Because the concentrations of these compounds typically increase during diapause and overwintering, the pool of bound water will increase while the the pool of free, osmotically active water (bulk water) will decrease. Increasing osmolality of body solutions will limit the avalibility of water molecules for evaporation and ice crystal formation (Block, 2002).

The regulation of ion concentrations across the cell membranes requires maintenance of the membrane integrity and active (ATP-dependent) pumping of ions. Failure to maintain specific ion concentrations inside and outside the cell may lead to severe metabolic perturbations (loss of excitability in nerves, inability to keep cell volume, failure of secondary transports, opening of voltage-dependent $Ca^{2+}$ channels, leakage of $Ca^{2+}$ from endoplasmic reticulum), cell disintegration and death (Hochachka, 1986). To avoid dissipation of membrane potentials and stabilize membrane function, cold hardy insects have evolved "channel arrest" adaptation, which prevents the ion leakage through the channels. The decreased ion flux then allows the conservation of energy by reducing the need for ATP-demanding ion pumping (Košťál et al., 2004c; Zachariassen et al., 2004).

Adjustments of cell membrane composition represent an important part of cold acclimation process. Our knowledge of compositional alteration of membrane lipids due to temperature changes has been emerging gradually, originating from early observations a century ago (Henriques & Hansen, 1901) and culminating in 1974 when the theory of *homeoviscous adaptation* (HVA) was formulated by Sinensky (1974). According to this theory, the general trend is an increase in unsaturated fatty acids at lower temperatures and an increase in saturated fatty acids at higher temperatures. HVA serves to maintain the correct membrane fluidity and its optimal functioning at the new conditions. This theory has been studied in a variety of organisms from bacteria to vertebrates, and its validity has been tested at both organismal (acclimatory) and evolutionary (adaptational) levels (Cossins & Prosser, 1978; Behan-Martin et al., 1993). Although HVA is the most often used paradigm to interpret the temperature-induced restructuring of membranes, some observations are

difficult to explain solely in terms of HVA. Thus, McElhaney (1984) introduced the term *homeophasic adaptation* (HPA) to stress the preservation of appropriate (liquid crystalline) phase for membrane functionality and later, Hazel (1995) developed and broadened the concept to *dynamic phase behavior* (DPB), to emphasize the dynamism of phase changes. In his model of DPB, the relationship between body temperature and transition temperatures of the lipid phases are conserved by acclimatory and adaptational adjustments of membrane composition. The adaptive meaning of DPB is to keep the physiologically required hexagonal II phases areas (for vesicular transports, regulation of activities of certain membrane enzymes), to prevent unregulated formation of disruptive hexagonal structures and to hold membrane lipids sufficiently "far" from the deleterious transition to gel phase. Acclimatory responses to cold causing restructuring of phospholipids in biological membranes have been shown in a few insect species, e.g. *Cymbalophora pudica* (Košťál & Šimek, 1998), *Drosophila melanogaster* (Overgaard et al., 2005), *Chymomyza costata* (Košťál et al., 2003), *P. apterus* (Hodková et al., 1999, 2002; Šlachta et al., 2002; Tomčala et al., 2006).

There are other known physiological mechanisms for insect cold hardiness. Antifreeze proteins (AFPs), ice-nucleating agents (INAs) or late embryogenesis abundant (LEA) proteins possess cryoprotective functions and are used by various insects to prevent cold injury (mainly ice crystal formation) (Ramsay, 1964; Patterson et al., 1981; Hew et al., 1983; Zachariassen & Hammel, 1976; Zachariassen, 1985; Goyal et al., 2005), but these were not the highlights of my studies, therefore they are not described more thoroughly.

## Low molecular mass cryoprotectants

The importance of low molecular mass cryoprotectants for overwintering survival of insects was recognized many years ago (Chino, 1957; Salt, 1957; Wyatt, 1963). Cryoprotectants known in insects include polyols (glycerol, sorbitol, ribitol, mannitol etc.), sugars (trehalose, fructose etc.), and also amino acids such as proline (Miller & Smith, 1975; Sømme, 1982; Lee & Denlinger, 1991). Their cryoprotective role is based on either colligative or non-colligative action. High concentrations of cryoprotectants (> 1 $mol.kg^{-1}$) will cause considerable colligative depression of melting and supercooling points in the freeze-avoiding insects. In the freeze-tolerant species, they will regulate the extent of cell dehydration caused by extracellular ice formation (Zachariassen, 1985; Lee & Denlinger, 1991). Non-colligative effects of polyols accumulated in low concentrations (typically tens to hundreds $mmol.kg^{-1}$) are probably based on stabilization and protection of functional structures of biological membranes and proteins (Crowe et al., 1987; Carpenter & Crowe, 1988; Storey & Storey, 1988, 1991; Sussich et al., 2001). It has been shown that low temperature (< 5°C) serves as a main factor triggering polyol biosynthesis and accumulation (Storey & Storey, 1991). In some insects, the ability to accumulate cryoprotectants is

restricted only to the individuals that have previously entered diapause (Šlachta et al., 2002), in other species, anaerobiosis or various environmental cues, such as photoperiod, food and water availability, can potently stimulate their formation (Meyer, 1978; Storey & Storey, 1988).

Cryoprotectant biosynthesis takes place in fat body tissue (except eggs) (Hayakawa & Chino, 1981) and glycogen reserves serve as the main source. Enzymatic complement involved in cryoprotectant biosynthesis is in the focus of my Thesis. In the following text, I have summarized the information available on structural and functional characterization of important enzymes and also gene expression of their coding genes.

*Glycogen phosphorylase (GPase)* catalyzes glycogenolysis which may increase the flow of carbon to the hexose monophosphate shunt (pentose cycle) and to metabolic pathways where sugars and polyols are synthetized (Storey & Storey, 1981). GPase is functionally active in a form of homodimer. In insect species, amino acid sequence has been described in e.g. *D. melanogaster* (Tick et al., 1999). In insect fat body, this enzyme exists in two forms, an inactive *b* form and an active *a* form and it is regulated both allosterically and hormonally. Active cAMP dependent phosphorylase kinase phosphorylates GPase *b*, converting into active GPase *a*, which then begins glycogen breakdown. GPase *a* is reconverted to GPase *b* by the hydrolysis of its phosphate by protein phosphatase-1. The stimulating role of low temperature (in the range of 0-5ºC) leading to GPase activation has been well documented in many insects (Ziegler et al., 1979; Hayakawa, 1985). Diverse regulation of phosphorylase kinase and phosphatase are involved in the activation of GPase by cold. Similar cold GPase activation has also been noticed in non-cold-hardy insect species (Ziegler et al., 1979). The percentage of GPase *a* form returns to a lower level when cryoprotectant content reaches its maximum (Churchill & Storey, 1989).

*Glucose-6-phosphate dehydrogenase (G6PDH)* is the first and key enzyme in pentose cycle, a metabolic pathway that supplies reducing energy to cells by maintaining the level of the co-enzyme nicotinamide adenine dinucleotide phosphate (NADPH). It catalyzes conversion of glucose-6-phosphate into 6-phosphogluconolacton. G6PDH functions as monomer in all cells. Fouts et al. (1988) characterized its nucleotide sequence in *D. melanogaster* and assessed 65% homology to human gene. They found out two isoforms of G6PDH that differ in a number of introns. Fractional sequences are known in other insect species: *Adalia decempunctata* or *A. bipunctata* (Jiggins, 2005)*, Ceratitis capitata* (Scott et al., 1993) and *P. apterus* (Košťál & Tollarová-Borovanská, unpublished data). Increasing activity of G6PDH with decreasing temperature observed in acclimated *P. apterus* suggests that the activity of pentose cycle is relatively elevated at low temperatures in diapausing insects and thus associated production of reducing power in the form of NADPH is critically needed for polyol synthesis (Košťál, et al., 2004b).

*Phosphofructokinase-1 (PFK-1)* is the most important regulatory enzyme of glycolysis. It is allosterically controlled by several activators and inhibitors. PFK-1 catalyzes one of the important steps of glycolysis, the conversion of fructose-6-phosphate and ATP to fructose-1,6-bisphosphate and ADP. Valaitis et al. (1992) have

purified tetramer 330kDa PFK-1 in *L. dispar*, significantly similar to the mammalian enzyme. A single study of Currie and Sullivan (1994) has resulted in 155kDa protein in *D. melanogaster*, which is approximately half the size of the mammalian and silk moth enzyme. In some insect species (e.g. *E. solidaginis*), producing both glycerol and sorbitol as cryoprotectants, PFK-1 regulation is pivotal. The enzyme must be active during glycerol synthesis at warm temperatures and inactive to elevate carbon flow to pentose cycle, where sorbitol synthesis occurs at low temperature (Storey & Storey, 1983). PFK-1 activity is manyfold higher in glycerol producing species than in species that form e.g. trehalose as a cryoprotectant (Hayakawa & Chino, 1982).

Next important regulatory enzyme of glycolysis might be *pyruvate kinase (PK)*, which catalyzes the transfer of a phosphate group from phosphoenolpyruvate to ADP, yielding one molecule of pyruvate and one molecule of ATP. This enzyme is allosterically regulated by products originated in glycolysis. In insects, PK has been sequenced in e.g. *D. melanogaster* (Hsiao et al., 2002)*, B. mori* (Sakano, unpublished data) or *P. apterus* (Košťál & Tollarová-Borovanská, unpublished data). In overwintering *E. solidaginis* low temperature acclimation resulted in unaffected activities of PK, that remained constant (Storey & Storey, 1981). Similar results in PK activity were observed in diapausing cold-acclimated adults of *P. apterus* (Košťál & Tollarová-Borovanská, unpublished data). Different situation has been found in *Chilo suppressalis,* where glycerol synthesis resulted from the inhibition of PK (Li et al., 2002).

Another enzymes of the Krebs cycle (e.g. *citrate synthese, CS*) or respiratory chain complex (e.g. *cytochrome c oxidase, COX*) might be subjects of studies in context to diapause and overwintering. Some partial sequences of CS have been identified in *D. melanogaster* and *Anopheles gambiae* (unpublished data). COX consists of 3 subunits (catalytic region) encoded by mitochondrial DNA and 7-10 subunits (modulating overall activity of the complex) encoded by nuclear DNA. Nucleotide sequence has been characterized in subunits I, II, III (de Bruijn, 1983) and $V_a$ (Caggese et al., 1999) in e.g. *D. melanogaster*. COX genes serve as a useful markers in phylogenetic studies separating species which morphological differences are subtle (Monti et al., 2005).

Less is known about the mitochondrial enzymes in terms of their activities or gene expression in context to overwintering. Decreased oxygen uptake could correlate with reduced oxidative metabolism in the arrested state. Work with *E. solidaginis* and *Epiblema scudderiana* indicated reduced mitochondrial function over the winter months, both species showing a significant reduction in the activities of following mitochondrial enzymes: CS, glutamate dehydrogenase, NAD-isocitrate dehydrogenase and COX (Joanisse & Storey, 1994). Such suppression of mitochondrial metabolism during winter could come from reduced numbers of mitochondria in the overwintering larvae or from reversible regulatory controls that inhibit mitochondrial function (McMullen & Storey, 2008). Uno et al. (2004) isolated COX subunit I in *Agrius convolvuli* species and observed some changes in abundance of its transcripts during pupal diapause. They detected dramatic increase of specific mRNA in the phase of diapause termination correlating with high activity of COX enzyme. This gene is thus up-regulated when diapause is terminating due to high oxygen utilization. Similar

findings were discovered during termination of diapause in larva of the moth *Omphisa fuscidentalis*. Activation of COX subunit I correlated with increase of COX enzyme activity, followed by an increase in oxygen consumption rate (Singtripop et al., 2007). Although metabolism in insects is typically suppressed in diapause, such suppression in adult stages is not as extensive, so the up-regulation of two mitochondrial COX subunits, I and III, found in early *C. pipiens* diapause may not be counterintuitive (Robich et al., 2007).

Apart from other enzymes involved in direct polyol biosynthesis reactions, *aldose reductase (AR), ketose reductase (KR)* or *polyol dehydrogenase (PDH)*, are in the centre of my interest. They convert sugars to sugar alcohols using NADPH or NADH as the reducing agents. AR or KR, constituents of the polyol pathway, belong to the aldo-keto reductase superfamily (AKR superfamily). The superfamily contains 115 NAD(P)(H)-dependent oxidoreductase proteins expressed in prokaryotes and eukaryotes that are distributed over 14 families (AKR1-AKR14). The current nomenclature and up-dated database is declassified on www.med.upenn.edu/akr web sites (Hyndman et al., 2003). PDH, is a member of medium-chain dehydrogenases/reductases superfamily (MDR superfamily). MDRs constitute of large group of about 1000 proteins. Within the MDR superfamily, at least eight families were distinguished. The most well known are alcohol dehydrogenases (ADH family) and polyol dehydrogenases (PDH family) (Nordling et al., 2002).

Additional data on the enzymatic control of polyol synthesis are fragmentary. Another sporadic research has been accomplished in glycerol producing species (*Protophormia terranovae, C. suppressalis*), where both *glycerol-3-phosphate dehydrogenase* and *glycerol-3-phosphatase* activities increased during diapause and overwintering (Wood et al., 1977; Li et al., 2002).

## Heat shock proteins

Heat shock proteins (Hsps) appear as promising candidates participating in mediation of insect cold hardiness and rapid temperature stress responses (Yocum et al, 1998; Yocum, 2001). The Hsps are represented by five major Hsp families: small Hsps, 60, 70, 90 and 100 kDa Hsps, separated according to their molecular mass (Georgopoulos & Welch, 1993; Liang & MacRae, 1997; Denlinger et al., 2001). They are found in all organisms and their structure and function are highly conserved (Schlesinger, 1990), therefore their popularity in molecular studies is large. The proteins are so named because they were initially discovered in fruit flies that were exposed to high heat (Tissières et al., 1974). But, Hsps are expressed in response to a variety of environmental stresses such as heat and cold shocks, osmotic and oxidative stresses, heavy metal exposure, irradiation, viral infection and high population densities (De Maio, 1999). The increase in Hsps expression is transcriptionally regulated (Wu, 1995). In addition to inducible forms, the Hsps also include constitutive forms – heat shock cognates. The inducible forms function as molecular

chaperones. They bind to proteins that were partially denatured during environmental stress and mediate either their repair or proteasomal degradation. The cognates promote correct folding of proteins after translation, translocation of proteins across membranes and also prevent the aggregation of proteins in an unstressed cell (Craig et al., 1994; Feder & Hofmann, 1999; Borges & Ramos, 2005).

Although it has been well established that Hsps participate in heat shock response, their roles during diapause development and cold exposure are still not well understood in insects. The most studied transcript is the inducible form of *hsp70*. Up-regulation of *hsp70* mRNA levels in response to low temperature was reported in several insect species: *Drosophila sp.* (Goto & Kimura, 1998), *L. decemlineata* (Yocum, 2001), *Delia antiqua* (Chen et al., 2006) and *Liriomyza huidobrensis* (Huang et al., 2007). Relatively high levels of mRNA transcripts for Hsps were also detected in diapause insects during overwintering (Rinehart et al., 2000; Hayward et al., 2005; Yocum et al., 2006) and in the larva of Antarctic midge, *Belgica antarctica* (Rinehart et al, 2006). Such observations led the authors to speculate on the role of Hsps in insect cold tolerance. Current evidence suggests, however, that mRNA levels provide little information on protein abundance and activity (Feder & Walser, 2005) and that detailed functional studies are needed to elucidate the influence of candidate genes on phenotype. Up-regulation of Hsps at a protein level was verified in the fruit flies exposed to cold (Burton et al., 1988; Sejerkilde et al., 2003), and in the pupae of flesh fly, *S. crassipalpiss* during diapause (Li et al., 2007). The most complex study has been done on the flesh fly *S. crassipalpis* (Joplin et al., 1990; Yocum et al., 1998; Rinehart et al., 2000; Hayward et al., 2005). They propose that up-regulation of Hsps during diapause or cold stress is a major factor contributing to cold-hardiness of overwintering insects. However, to our knowledge, the only direct evidence obtained so far for positive role of heat shock proteins in insect cold tolerance is that by Rinehart et al. (2007) who injected the *hsp23* and *hsp70* dsRNAs (double-strand RNAs) into the pre-diapause larvae of *S. crassipalpis*, and observed dramatic decrease of mRNA levels in pupae and a significant loss of their cold tolerance.

## Cold acclimation and cold hardiness in *P. apterus*

Adults of *P. apterus* enter into facultative reproductive diapause before overwintering. Diapause is an essential prerequisite for their succesful overwintering as it allows the process of autumnal acclimatization to proceed. Thus, the level of chill tolerance is much higher in diapausing than in non-diapausing (reproducing) individuals of *P. apterus* (Šlachta et al., 2002). Diapausing adults overwinter in a supercooled state and do not tolerate freezing of their body fluids. They decrease their supercooling point (SCP, temperature of spontaneous ice crystalization) down to the minimum between -16°C and -21°C (Hodková & Hodek, 1997; Košťál & Šimek, 2000).

Very high amounts of nutrient reserves, in the form of glycogen, triglycerides and hexameric proteins, were found in the fat body and haemolymph of diapausing individuals (Šula et al., 1998; Košťál et al., 2004a). Glycogen stores are considered to serve as the principal source of carbon for polyol biosynthesis at low temperatures (Storey & Storey, 1991). In accordance with this view, the glycogen reserves are depleted during cold-acclimation (Košťál et al., 2004a).

No information is available about the potential mechanisms preventing water evaporation or ice penetration through the cuticle. The process of cold-acclimation, however, is accompanied with partial dehydration (loss of approximately 10% of body water), which affects primarily the haemolymph compartment. Thus, the body water is partially redistributed and a 'reserve' of hypo-osmotic fluid accumulates in the hindgut. Diapausing cold acclimated adults of *P. apterus* show a good ability to maintain ion gradients across cell membranes when exposed to subzero temperatures (Košťál et al., 2004c).

Field and laboratory experiments were conducted to study changes in membrane composition during overwintering. Changes in relative proportions of major molecular species of glycerophosphoethanolamines (GPEtns) and glycerophosphocholines (GPChols) in two different tissues (fat body and thoracic muscle) were followed. The relative proportion of total GPEtns increased, while the proportion of total GPChols decreased during autumnal acclimatization in the field. The relative proportion of unsaturated fatty acyls slightly decreased. A similar restructuring response was seen during cold-acclimation in the field and in the laboratory (Hodková et al., 1999; Tomčala et al., 2006).

Mechanisms of antioxidant defence have not been studied in *P. apterus*. Protective components such as AFPs have not been found in this species (Košťál, unpublished results). Since *P. apterus* belongs to freeze intolerant species, INAs are not produced. No evidence of presence of LEAs has been provided yet.

The seasonal increase of cold tolerance coincides in time with the accumulation of four "winter" polyols (ribitol, sorbitol, mannitol, arabinitol) in diapausing *P. apterus* adults (Košťál & Šimek, 2000). The accumulation is triggered by ambient temperatures below a threshold of 5°C (Košťál et al., 2001). A tight relationship between total concentration of winter polyols and cold hardiness has been revealed (Košťál & Šlachta, 2001). Relatively low concetrations of polyols (without any significant colligative effect on SCP) have been found to be sufficient to enhance survival at sub-zero temperatures (Košťál et al., 2001). Metabolic adjustments for polyol biosynthesis in *P. apterus* and transcription of genes coding for polyol biosynthetic enzymes are presented in this Thesis in detail (Košťál et al., 2004a/b; Tollarová, 2008; Košťál et al., 2008).

Hsps in context to temperature stress in the adult *P. apterus* have not been studied so far and our results bring the first data (Košťál & Tollarová-Borovanská, 2009).

# Aims of research

1. To conduct a detailed physiological and biochemical study focused on the process of polyol biosynthesis and accumulation in diapausing adults of *Pyrrhocoris apterus*. To asses the building and degradation of glycogen reserves, the activities of selected enzymes involved in energy and polyol metabolism, and the levels of winter polyols in variously acclimated adults of *P. apterus*.

2. To clarify the triggering mechanisms for seasonal restructuring of membrane phospholipids in the overwintering adults of *P. apterus.*

3. To bring new insights into the diapause physiology of *P. apterus* by employing the molecular methods. To clone and sequence the genes coding for proteins implemented in energy metabolism (citrate synthase, cytochrome c oxidase), polyol cryoprotectant biosynthesis (aldose reductase, polyol dehydrogenase), and stress response (70 kDa heat shock proteins) and to study their regulation in relation to diapause and cold-acclimation.

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*Physiological Entomology* (2004) **29**, 344–355

# Enzymatic capacity for accumulation of polyol cryoprotectants changes during diapause development in the adult red firebug, *Pyrrhocoris apterus*

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**Abstract.** Diapausing adults of the red firebug, *Pyrrhocoris apterus*, were maintained in the laboratory at constant conditions of 20 °C and short days (LD 12 : 12 h) for 5 months. Spontaneous termination of diapause is seen in 0.4% of adults. At different times of diapause development, groups of adults were exposed to an 8-week gradual cold treatment ending at 0 °C. Ribitol and sorbitol contents remain very low at constant 20 °C and their rapid accumulation only occurrs at temperatures below 5 °C. The capacity to accumulate ribitol in response to low temperature stimulus remains relatively stable but the capacity to accumulate sorbitol decreases to zero during the 5 months of diapause development. Glycogen (whole-body and fat-body reserves) accumulates relatively rapidly during the early phase (up to 1–2 months) and slowly depletes during the late phase of diapause development under constant conditions. Upon cold treatment, part of the glycogen reserve was depleted. The activities of most enzymes involved in polyol metabolism (namely glycogen phosphorylase, glucose-6-P dehydrogenase, phosphofructokinase, aldose reductase, polyol dehydrogenase and ketose reductase) increase relatively rapidly during the early phase of diapause development and, in the late phase, they either become stable or slowly decrease. Cold treatment has either no effect or results in a moderate increase in activity when applied in the early phase of diapause development but results in a more or less obvious decrease of enzymatic activity when applied in the late phase.

**Key words.** Cold hardiness, diapause, glycogen, Heteroptera, metabolic enzymes, ribitol, sorbitol.

Journal of Insect Physiology 50 (2004) 303–313

Journal of Insect Physiology

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# Adjustments of the enzymatic complement for polyol biosynthesis and accumulation in diapausing cold-acclimated adults of *Pyrrhocoris apterus*

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Received 17 October 2003; received in revised form 8 January 2004; accepted 8 January 2004

**Abstract**

The capacity to accumulate winter polyols (mainly ribitol and sorbitol) during cold-acclimation in *Pyrrhocoris apterus* is restricted only to the adults that have previously entered diapause. The enzymatic complement involved in polyol biosynthesis was found to differ in a complex manner between diapause and non-diapause adults. Nearly 100% of glycogen phosphorylase (*GPase*) was present in its active form in non-diapause adults irrespective of their acclimation status. In contrast, less than 40% of *GPase* was present in its active form in diapause adults prior to cold-acclimation and the inactive form was rapidly activated upon transition from 5 to 0 °C, concomitantly with the start of rapid polyol accumulation. The flow of carbon released by activation of glycogen degradation might be routed to the pentose cycle because the activity of glucose-6-P dehydrogenase (*$G_6P$-DH*) was significantly higher and it increased with cold-acclimation in diapause adults while it was relatively low and it decreased with cold-acclimation in non-diapause adults. Reducing equivalents in the form of NADPH, which were generated in the pentose cycle, might require re-oxidation. Such re-oxidation might be achieved during reduction of sugars to polyols. The activity of NADP(H)-dependent aldose reductase (*AR*) was about 20-fold higher in diapause than in non-diapause adults. Similarly, the activity of NAD(H)-dependent polyol dehydrogenase (*PDH*) was higher in diapause adults. In addition, we found a very high activity of an unusual enzyme, NADP(H)-dependent ketose reductase (KR), exclusively in diapause adults. *KR* might be involved in reduction of fructose to sorbitol. Although its affinity for fructose as a substrate was low ($K_M = 0.64$ M), its activity was about 10-fold higher than that of *PDH* with fructose. Moreover, the activity of *KR* significantly increased with cold-acclimation while that of *PDH* remained unchanged. Different electrophoretic mobilities in PAGE gel suggested that *KR* and *PDH* are two different enzymes with specific requirement for NADP(H) or NAD(H), respectively, as co-factors.


*Keywords:* Diapause; Cold tolerance; Cryoprotectans; Ribitol; Sorbitol; Glycolysis; Pentose cycle; Ketose reductase

The Journal of Experimental Biology 209, 4102-4114
Published by The Company of Biologists 2006
doi:10.1242/jeb.02484

# Seasonal acquisition of chill tolerance and restructuring of membrane glycerophospholipids in an overwintering insect: triggering by low temperature, desiccation and diapause progression

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*Accepted 10 August 2006*

## Summary

**Adults of the insect *Pyrrhocoris apterus* acquire chill tolerance through the process of autumnal acclimatization. Field and laboratory experiments were conducted to separate the triggering effects of low temperatures, desiccation and diapause progression on the physiological characteristics related to chill tolerance with emphasis on the restructuring of glycerophospholipid (GPL) composition. Changes in relative proportions of major molecular species of glycerophosphoethanolamines (GPEtns) and glycerophosphocholines (GPChols) in thoracic muscle and fat body tissues were followed using HPLC coupled to electrospray ionisation mass spectrometry. The increase in relative proportion of 1-palmitoyl-2-linoleyl-*sn*-GPEtn at the expense of 1,2-dilinoleyl-*sn*-GPChol was the most prominent feature of the complex change observed in both tissues during autumnal acclimatization in the field. The relative proportion of total GPEtns increased, while the proportion of total GPChols decreased. The relative proportion of unsaturated fatty acyls slightly decreased. A similar restructuring response was seen during acclimatization in the field and cold acclimation in the laboratory. By contrast, the GPL changes related to desiccation and diapause progression were relatively small, differed qualitatively from the cold-acclimation response, and were accompanied with no increase of chill tolerance. Other features of autumnal acclimatization, i.e. depression of supercooling capacity and accumulation of polyhydric alcohols, were also triggered solely by low temperatures.**

Key words: membrane phospholipids, temperature, acclimatization, cold acclimation, desiccation, diapause, cold tolerance, ectotherm, Insecta, Heteroptera.

NOTE

*Eur. J. Entomol.* **105**: 149–152, 2008
http://www.eje.cz/scripts/viewabstract.php?abstract=1314
ISSN 1210-5759 (print), 1802-8829 (online)

# Seasonal activity-profiles of enzymes involved in cryoprotectant biosynthesis in *Pyrrhocoris apterus* (Heteroptera: Pyrrhocoridae)

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**Key words.** Diapause, overwintering, metabolism, enzymes, polyols, Heteroptera, *Pyrrhocoris apterus*

**Abstract.** The activities of three enzymes involved in polyol biosynthesis (aldose reductase, AR; ketose reductase, KR; and polyol dehydrogenase, PDH) were studied in adult females of the linden bug, *Pyrrhocoris apterus*, collected from the field during 2005/2006. While the activities of three enzymes were low in reproductive females, activities greater by one or two orders were seen in reproductively arrested females. AR and KR showed similar seasonal trends in activity. Activities were low during diapause initation and later increased and stabilized during autumnal diapause development. Further increases of AR and KR activities were seen during low temperature quiescence and finally the activities sharply decreased during vernal resumption of direct development. The activity of PDH was relatively high (but fluctuating) during diapause, then decreased in quiescent insects and almost disapeared in reproductively active females. Insects collected in February were subjected to laboratory de-acclimation (exposure to high temperatures) followed by re-acclimation (exposure to low temperatures) which resulted in loss of activity in all three enzymes and no regain. High activities of AR, KR and PDH in reproductively arrested females thus conform well with their previously observed high capacity to synthesize and accumulate polyol cryoprotectants.

Available online at www.sciencedirect.com
ScienceDirect
Journal of Insect Physiology 54 (2008) 77–88
Journal of Insect Physiology
www.elsevier.com/locate/jinsphys

# Dynamism in physiology and gene transcription during reproductive diapause in a heteropteran bug, *Pyrrhocoris apterus*

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Received 2 July 2007; received in revised form 9 August 2007; accepted 9 August 2007

**Abstract**

Reproductive diapause was characterized in females of *Pyrrhocoris apterus* using physiological parameters (diapause intensity, photoperiodic responsiveness, oxygen consumption, mass and hydration) and changes in relative abundance of mRNA transcripts of eight different genes coding for proteins implemented in energy metabolism, cryoprotectant biosynthesis, biological clocks, and hormonal receptors. Changes in diapause intensity served as a basis for distinguishing successive phases of diapause development, which were driven both endogenously (under constant environmental conditions) and exogenously (in response to a change in environmental conditions). Changes in the relative levels of transcripts of genes coding for aldose reductase (*AR*) and sorbitol dehydrogenase (*SoDH*) closely matched those of diapause intensity and thus appeared as promising molecular markers of diapause and its development. During the initiation phase, the intensity of diapause and the levels of *AR* and *SoDH* transcripts increased and reached a maximum. During maintenance, under a constant temperature of 20 °C and short-day photoperiod, the intensity of diapause and the levels of both transcripts first decreased and, later, were maintained constant. Termination of diapause was stimulated by cold, during which the intensity of diapause and the levels of both transcripts further decreased. Upon resumption of direct development (oogenesis, mating and oviposition), the relative abundances of *AR* and *SoDH* transcripts decreased to trace levels.


*Keywords:* Photoperiodism; Development; Diapause intensity; Reproduction; Gene transcription

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PLoS one

# The 70 kDa Heat Shock Protein Assists during the Repair of Chilling Injury in the Insect, *Pyrrhocoris apterus*

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## Abstract

***Background:*** The *Pyrrhocoris apterus* (Insecta: Heteroptera) adults attain high levels of cold tolerance during their overwintering diapause. Non-diapause reproducing adults, however, lack the capacity to express a whole array of cold-tolerance adaptations and show relatively low survival when exposed to sub-zero temperatures. We assessed the competence of non-diapause males of *P. apterus* for responding to heat- and cold-stresses by up-regulation of 70 kDa heat shock proteins (Hsps) and the role of Hsps during repair of heat- and cold-induced injury.

***Principal Findings:*** The fragments of *P. apterus* homologues of Hsp70 inducible (PaHsp70) and cognate forms (PaHsc70) were cloned and sequenced. The abundance of mRNA transcripts for the inducible form (qPCR) and corresponding protein (Western blotting) were significantly up-regulated in response to high and low temperature stimuli. In the cognate form, mRNA was slightly up-regulated in response to both stressors but very low or no up-regulation of protein was apparent after heat- or cold-stress, respectively. Injection of 695 bp-long *Pahsp70* dsRNA (RNAi) caused drastic suppression of the heat- and cold-stress-induced *Pahsp70* mRNA response and the up-regulation of corresponding protein was practically eliminated. Our RNAi predictably prevented recovery from heat shock and, in addition, negatively influenced repair of chilling injuries caused by cold stress. Cold tolerance increased when the insects were first exposed to a mild heat shock, in order to trigger the up-regulation of PaHsp70, and subsequently exposed to cold stress.

***Conclusion:*** Our results suggest that accumulation of PaHsp70 belongs to a complex cold tolerance adaptation in the insect *Pyrrhocoris apterus*.

**Citation:** Koštál V, Tollarová-Borovanská M (2009) The 70 kDa Heat Shock Protein Assists during the Repair of Chilling Injury in the Insect, *Pyrrhocoris apterus*. PLoS ONE 4(2): e4546. doi:10.1371/journal.pone.0004546

**Editor:** Marcelo Hermes-Lima, Universidade de Brasília, Brazil

**Received** October 16, 2008; **Accepted** January 9, 2009; **Published** February 20, 2009



**Funding:** This study was supported by the Czech Science Foundation Grant No. 206/07/0269. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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

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# INSECT COLD TOLERANCE AND REPAIR OF CHILL-INJURY AT FLUCTUATING THERMAL REGIMES: ROLE OF 70 kDa HEAT SHOCK PROTEIN EXPRESSION.

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## Abstract

Expression of heat shock proteins has been proposed as an underlying mechanism of increased cold tolerance in insects exposed to fluctuating thermal regimes (FTRs) in comparison to constant low temperatures (CLTs). We found that the levels of *Pahsp70* mRNA increase by up to 3 orders in the linden bugs, *Pyrrhocoris apterus* exposed to FTR -5ºC (22h)/ 25ºC (2h). The 2h-long warm pulses, however, were not sufficient for accumulation of PaHSP70 protein and thus no significant difference in expression of PaHSP70 protein was detected between FTR and CLT regimes. Hence, we conclude that the accumulation of PaHSP70 protein is not the mechanism underlying the increased cold tolerance in *P. apterus* at the particular FTR used in this study. The relevance of some other possible mechanisms is discussed.

**Keywords:** insect; cold tolerance; heat shock proteins; fluctuating thermal regime

# Summary of results

**1. Enzymatic capacity for accumulation of polyol cryoprotectants changes during diapause development in the adult red firebug, *Pyrrhocoris apterus***

- Glycogen stores were rapidly accumulated during the early phase and slowly depleted during the late phase of diapause development. Reduction of glycogen stores during the cold treatment was relatively constant throughout the whole diapause.
- Except *phosphofructokinase-1* (PFK-1), all studied enzymes (*glycogen phosphorylase,* GPase; *glucose-6-phosphate dehydrogenase,* G6PDH; *polyol dehydrogenase,* PDH; *aldose reductase,* AR; and *ketose reductase,* KR) displayed a rapid increase in activity during the early phase and, during the late phase, activities remained stable or slowly decreased. The activity of PFK-1 was either stable or moderately increased with diapause progression. In response to cold treatment: (i) the activity of *a* form of GPase decreased; (ii) no change in G6PDH activity was observed; and (iii) the activities of PDH, AR and KR decreased.
- No polyols were accumulated during diapause development at high temperatures. Rapid accumulation of polyols appeared in temperatures below a threshold of about 5ºC.

**2. Adjustments of the enzymatic complement for polyol biosynthesis and accumulation in diapausing cold-acclimated adults of *Pyrrhocoris apterus***

- No significant changes were found in the content of fat body glycogen in both diapausing or non-diapausing bugs during cold-acclimation. In non-diapausing adults, the glycogen content was much lower than in diapausing individuals.
- Activities of GPase (total) and PFK were similar in non-diapause and diapause specimens. Almost 100% of GPase was present in its active *a* form in non-diapausing adults. In diapausing adults, the GPase inactive *b* form (about 60%) was activated upon transition to 0ºC, which probably coincided with the accumulation of polyols at such sub-threshold temperature. Profound

differences were detected between non-diapause and diapause specimens in the activities of G6PDH, AR and PDH. The activity of KR, was observed exclusively in the diapausing individuals. $K_M$ values of PDH and KR and their electrophoretic mobilities in native polyacrylamide gel differed clearly.

- The capacity to accumulate winter polyols during cold-acclimation was restricted to the adults that have previously entered diapause. No polyol accumulation was observed in non-diapausing group.

**3. Seasonal acquisition of chill tolerance and restructuring of membrane glycerophospholipids in an overwintering insect: triggering by low temperature, desiccation and diapause progression**

- Relative proportion of glycerophosphoethanolamines increased, while the proportion of glycerophosphocholines decreased during autumnal acclimatization in the field and cold acclimation in the laboratory. The relative proportion of unsaturated fatty acyls slightly decreased.
- Overall glycerophospholipid changes after desiccation and diapause progression were relatively small.

**4. Seasonal activity-profiles of enzymes involved in cryoprotectant biosynthesis in *Pyrrhocoris apterus***

- Activities of enzymes involved in polyol biosynthesis (AR, KR and PDH) were low in reproductive adults. By contrast, greater activities were observed in reproductively arrested individuals.
- AR and KR showed similar seasonal trends: activities were low during diapause initiation, then increased and stabilized during further phases, another rapid increase was seen during low temperature quiescence and the activities sharply decreased during resumption of active development.
- PDH activity was high during diapause, then decreased in quiescent bugs and almost disappeared in insects with resumed development.
- Clear relationship between high activities of AR, KR and PDH and the capacity to accumulate polyols in only diapausing *P. apterus* was supported.

**5. Dynamism in physiology and gene transcription during reproductive diapause in a heteropteran bug, *Pyrrhocoris apterus***

- Dynamism of diapause development was characterized using (i) physiological parameters: time to oviposition, photoperiodic responsiveness, oxygen consumption, mass and hydration; and (ii) changes in transcription of genes involved in energy metabolism, cryoprotectant biosynthesis, biological clocks and hormonal receptors.
- Genes coding for AR and PDH appeared to be promising molecular markers of diapause development.
- Changes in diapause intensity served as a basis for recognizing successive phases of diapause.

**6. The 70 kDa heat shock protein assists during the repair of chilling injury in the insect, *Pyrrhocoris apterus***

- The fragments of *P. apterus* homologues of Hsp70 inducible (PaHsp70) and cognate forms (PaHsc70) were cloned and sequenced.
- PaHsp70 were significantly up-regulated in response to high and low temperature stimuli. PaHsc70 was slightly up-regulated in response to both stressors, but very low or no up-regulation of protein was apparent after heat- or cold-stress.
- RNAi (injection of *Pahsp70* ds RNA) caused drastic suppression of the heat- and cold-stress-induced *Pahsp70* mRNA response and the up-regulation of corresponding protein was practically eliminated.
- Accumulation of PaHsp70 probably belongs to a complex cold tolerance adaptation in the insect *P. apterus*.

**7. Insect cold tolerance and repair of chill-injury at fluctuating thermal regimes: role of 70kDa heat shock protein expression**

- Survival significantly increased when the insects were exposed to a fluctuating thermal regime (FTR) in comparison to a constant low temperature (CLT).

- *Pahsp70* mRNA levels were up-regulated during FTR. In contrast, no significant difference in expression of PaHSP70 protein was detected.
- Regulation of HSP70 protein levels were shown to play insignificant role in the FTR response of *P. apterus*.

# Prospects for future research

The studies on polyol cryoprotectants and heat shock proteins that have been successfuly extended or started, respectively, during the work on my doctoral Thesis will continue.

**Polyol cryoprotectants**

We plan to separate the proteins extracted from the fat body of acclimated adults of *P. apterus* using the 2D gel electrophoresis. Further, we will detect the protein spot with ketose reductase (KR) activity by the previously used technique (Košťál et al, 2004b) and sequence the protein. Protein sequence will be used to target the respective gene. After cloning and sequencing the KR gene, we wish to express it in a suitable bacterial or fungal vector and verify the KR activity in the protein product.

The double stranded RNAs against the gene transcripts coding for polyol dehydrogenase (PDH); aldose reductase (AR) and ketose reductase (KR) will be synthesized and used for RNAi study. We hope to bring a genetic proof for the importance of ribitol and sorbitol accumulation in the development of cold hardiness. After injecting the dsRNA into the diapause adults of *P. apterus* we plan to study the effects on: (a) the gene transcription; (b) enzymatic activities; (c) accumulation of ribitol and sorbitol; and (d) cold hardiness.

**Heat shock proteins**

The main objective of our ongoing study is to describe potential roles of the 70kDa heat shock proteins in diapausing adults of *P. apterus*. We will focus on changes of gene transcription (qRT-PCR) and protein abundance (ELISA) during the diapause

development , and on the relative importance of Hsps in the cold tolerance of diapausing adults, which display many other potent mechanisms of cold hardiness.

Our specific aims are to: (a) distinguish between the roles played by inducible and cognate forms of 70 kDa HSPs; (b) clone, sequence and study the roles of HSPs belonging to other families (small HSPs, 90 kDa HSPs); (c) use RNAi technique as a tool to reveal the functional aspects of HSPs upregulation.

# ABSTRACT 1

**Roles of diapause termination and cold-acclimation in cryoprotectant biosynthesis and accumulation in *Pyrrhocoris apterus* (Heteroptera)**.

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Adults of the red firebug, *Pyrrhocoris apterus* accumulate specific "winter" polyols (mainly ribitol and sorbitol) at a total concentration about 100 mM when exposed at temperatures below 5°C. No capacity for accumulation of winter polyols was detected in the non-diapause adults.

In this study we extend our previous results and show that the diapause and non-diapause adults differ in several important aspects of their fat body enzymatic complement: (1) while 95% of the glycogen phosphorylase (*GPase*) is present in the active *a* form in non-diapause adults, it is only 40 % in diapause adults. Inactive *b* form of *GPase* is rapidly activated upon exposure of diapause adults at 0°C. (2) Activity of glucose-6-P dehydrogenase ($G_6P$-*DH*) is 3 times higher in diapause than in non-diapause adults. This indicates relatively higher carbon flow through the hexose monophosphate shunt and higher production of reducing power in the form of NADPH in diapause adults. (3) NADPH-dependent aldose reductase (*AR*) is about 20 times higher in diapause than in non-diapause adults. *AR* preferentialy converts ribose to ribitol. (4) NADH-dependent polyol dehydrogenase (*PDH*) is about 12 times higher in diapause than in non-diapause adults. (5) An unusual enzymatic activity, NADPH-dependent ketose reductase/polyol dehydrogenase (*KR/PDH*) was detected exclusively in diapause adults. Such activity was very high and, together with the activity of conventional *PDH*, might have been responsible for accumulation of sorbitol. Analysis of $K_m$ values for specific substrates and visualization of specific activities on PAGE gels using tetrazolium salt methodology confirmed that *PDH* and *KR/PDH* are different enzymes and that *KR/PDH* activity is specific for diapause adults only.

TEMP 2003, International Symposium on Animal and Plant Cold Hardiness, České Budějovice, Czech Republic, August 10-15, 2003.

# ABSTRACT 2

**Activities of enzymes for cryoprotectant biosynthesis and transcription of their genes: roles of developmental programme and acclimation state.**

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Many overwintering insects accumulate sugars and polyols in their tissues, which serve to mitigate the impact of low temperatures. The peaking cryoprotectant concentrations usually coincide with the most cool season in the field and the low temperature was considered as a main stimulus for accumulation. Here we show that a developmental trigger is necessary to allow polyol accumulation, in addition to temperature stimulus, in the adult bugs, *Pyrrhocoris apterus*. Comparing responses in field-collected and laboratory-reared insects we found that: (a) activities of two key enzymes, which participate in the final reduction of sugars (glucose, fructose, ribose) to their respective polyols (sorbitol, ribitol), namely NADP(H)-dependent aldo-keto reductase (AKR) and NADH-dependent polyol dehydrogenase (PDH), markedly differ according to the developmental programme. The activities are much higher in diapausing than in reproducing insects; (b) the activities of AKR and PDH increase during cold-acclimation of diapausing insects; (c) the activities differ between reproducing insects that passed through diapause (overwintered generation) and those without diapause (spring generation); (d) the transcription rates of *PyrAKR* and *PyrPDH* genes are much higher in diapausing than in reproducing insects. Such results indicate that preparative steps (the increases of gene transcription rates and of abundance of enzyme molecules) must proceed at relatively high temperatures, during the onset of diapause developmental programme, which later allows accumulation of cryoprotectants upon exposure to low temperatures.

The First International Symposium on the Environmental Physiology of Ectotherms and Plants, Roskilde, Denmark, July 11 -16, 2005.

# Activities of enzymes for cryoprotectant biosynthesis and transcription of their genes: roles of developmental programme and acclimation state

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## Introduction

Many overwintering insects accumulate sugars and polyols in their tissues, which serves to mitigate the impact of low temperatures. The peaking cryoprotectant concentrations usually coincide with the most cool season in the field and the low temperature was considered as a main stimulus for accumulation. Here we show that a developmental trigger is necessary to allow ribitol and sorbitol accumulation, in addition to temperature stimulus, in the adult bugs of *Pyrrhocoris apterus*.

## Materials and Methods

Responses were compared in the insects (only females were used) that were either reared in laboratory under defined environmental conditions or collected in the field in different times of the year.

- Enzymatic activities of aldo/keto reductase (AKR) and polyol dehydrogenase (PDH) were measured in the female fat bodies (8 tissues per sample, 3 replicates) by continual scanning of absorbance at 340 nm [NAD(P)$^+$] → [NAD(P)H] on Pye Unicam SP8/100 spectrophotometer. Activities were expressed as mmoles of substrate converted to product during one minute per one gram of total tissue proteins (assessed by BCA method)

- Electrophoretic separation of fat body proteins was performed on gradient (4 – 20%) native PAGE gels. Bands containing AKR and PDH activities were visualized using chromogenic reaction of tetrazolium salts with reduced NAD(P)H cofactors that were produced during the enzymatic reactions with specific substrates.

- Partial nucleotide sequences of cDNAs coding for *PyrAKR* and *PyrPDH* genes were obtained by the standard RT-PCR method using degenerated primers. PCR cDNA products were digested with restriction enzymes and cloned into the pGEM-TEasy vector. The positive clones were cultured, their plasmid DNA was extracted and sequenced on ABI Prism 377 DNA Sequencer.

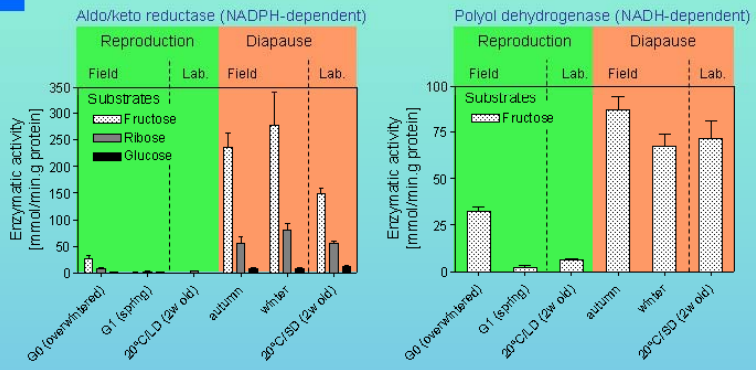
- Quantitation of the abundance of mRNA transcripts for *PyrAKR* and *PyrPDH* genes in the fat body tissue was achieved by using RT-PCR reactions with the gene specific primer pairs. The abundance of mRNA transcripts of *RP49* gene was used as standard. The number of PCR cycles was changed from 12 – 30 using Biometra T3000 cycler equipped with three independent blocks.

## Results

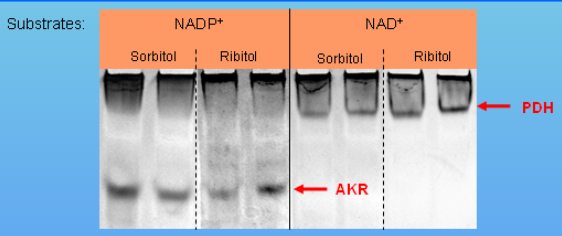
### 1. Activities of AKR and PDH are much higher in the diapausing than in the reproducing females

In both, the field-collected and laboratory-reared insects, we found that:

- the activities of two key enzymes, which participate in the final reduction of sugars (glucose, fructose, ribose) to their respective polyols (sorbitol, ribitol), were much higher in diapausing than in reproducing insects
- the activities of AKR and PDH increased during cold-acclimation of diapausing insects (Kostal et al., JIP 50, 303-313, 2004)
- the activities differed between reproducing insects that passed through diapause (overwintered G0 generation) and those without diapause (spring G1 generation)




### 2. AKR and PDH activities in diapausing females were confirmed by specific visualization following the electrophoretic separation of fat body proteins




"Positive" visualization reactions are shown in the Figure [polyol → sugar, coupled with NAD(P)$^+$ → NAD(P)H]. Reduced cofactors entered the cascade of redox reactions ending with the formation of blue precipitate, formazan (visible bands). Single band corresponding to AKR (or PDH) activity appeared after the reaction of substrates with NADP$^+$ (or NAD$^+$, respectively). No bands were detected when specific substrates were omitted.

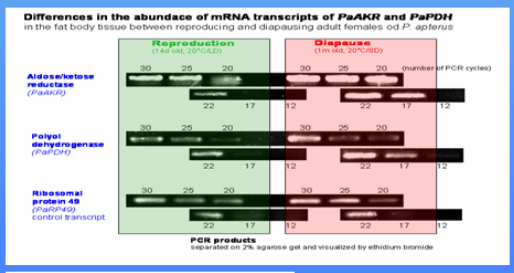
"Negative" visualization reactions [sugar → polyol, coupled with NAD(P)H → NAD(P)$^+$] are not shown. Single band coresponding to AKR activity appeared with all three sugar substrates (glucose, fructose, ribose) but we failed to detect any band corresponding to PDH activity. This analysis was confounded by the presence of non-specific reactions/bands.

### 3. Fragments of nucleotide sequences of PyrAKR and PyrPDH genes were obtained

The 381 bp-long fragment of *PyrAKR* was sequenced and translated into the amino acid (aa) sequence. Comparing the aa-translation with the known sequence in human *AKR* revealed that 63% of the aa are identical in both genes (80% share functional homology).

The 1020 bp-long fragment (including the 3' end) of *PyrPDH* was sequenced and translated into the amino acid (aa) sequence. Comparing the aa-translation with known sequences in *Bemisia argentifolii*, *Aedes aegypti* and *Drosophila melanogaster* revealed that 65.4, 66.6 and 63.9%, respectively, of the aa are identical. The regions required for catalytic activity of PDH were 100% conserved. *PyrPDH* clearly belongs to the MDR superfamily.

### 4. The abundances of mRNA transcripts for PyrAKR and PyrPDH genes are higher in the diapausing than in the reproducing females

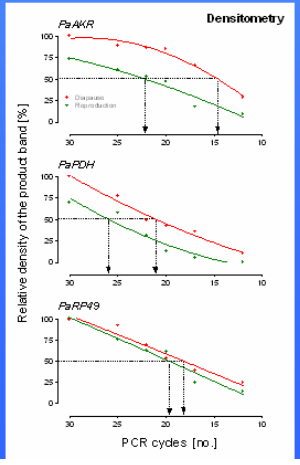



## Conclusions

- Although the accumulation of polyol cryoprotectants in *P. apterus* (and many other insects) starts only upon the stimulation by low temperatures, the capacity for accumulative biosynthesis establishes already during early diapause, at relatively high temperatures.

- Increased activities of polyol biosynthetic enzymes (AKR, PDH) and higher abundances of mRNA transcripts of their coding genes are characteristic features of the diapause syndrome in *P. apterus*.

- Laboratory-reared and field-collected insects did not significantly differ in AKR and PDH activities. Nevertheless, statistically significant differences were found between G0 and G1 generations collected in the field. It indicates that certain symptoms of the diapause syndrom (such as the increased AKR and PDH activities) may "outlive" the end of diapause and persist until spring resumption of reproductive activity.




Gene specific primers were designed based on sequenced fragments and used for preliminary quantitation of mRNA transcripts in the fat body tissues of diapausing and reproducing females. PCR products were separated on 2% agarose gel and stained by ethidium bromide. Single bands of expected size were found (*AKR*, 638 bp; *PDH*, 309 bp; *RP49*, 138 bp).

Densitometric analysis was performed using the QuantiScan software. The difference of ca. 8 cycles represents 256-fold difference in the abundance of *PaAKR* mRNA transcripts. 5 cycles correspond to the 32-fold difference (*PaPDH*) and 1 cycle corresponds to the 2-fold difference (*PaRP49*).

# ABSTRACT 3

**Reparation of heat and cold injury in the bug *Pyrrhocoris apterus*: does it require the expression of inducible *hsp70* gene?**

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**Introduction:**The expression of Heat Shock Proteins (Hsps) is known to be induced by diverse stresses, including heat and cold shocks, desiccation, anoxia, and exposure to a wide range of chemicals. Hsps function as molecular chaperones binding to partially denatured proteins and promoting their return to native conformation. Although it has been well established that Hsps are developmentally up-regulated in various diapausing insects, their exact physiological roles during diapause are not completely clear.

**Methods:** We have cloned structural homologs of inducible *hsp70* and cognate *hsc70* genes in the heteropteran *Pyrrhocoris apterus*, and quantified the abundance of their mRNA transcripts after the exposures to high (+45ºC) and low (-5ºC) temperature shocks using qRT-PCR technique.

**Results:** While the levels of *hsp70* mRNA increased by about three orders (1 000 – 4 000-fold) after the shocks, the levels of *hsc70* mRNA remained constant. After the injection of synthetic *hsp70* dsRNA (RNAi technique), we managed to diminish the shock-induced *hsp70* response to approximately 30 – 60-fold increase. Such a suppression of *hsp70* expression was sufficient to completely prevent the recovery from heat-injury. While more than 95 % of the control bugs (treated by injection buffer only) recovered and were fit 3d after the exposure, none of the *hsp70* dsRNA-treated bugs survived after the exposure to +45 ºC /5h.

**Conclusion:** The results of ongoing experiments will be presented, in which we asked whether the recovery of *P. apterus* bugs after cold shock also requires the up-regulated expression of inducible *hsp70* gene.

XXIII[rd] International Congress of Entomology, Durban, South Africa, July 6-12, 2008.

# Reparation of heat and cold injury in the bug *Pyrrhocoris apterus*: does it require the expression of inducible *hsp70* gene?

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## INTRODUCTION

Heat Shock Proteins (Hsps) are expressed in response to a variety of environmental stresses such as heat and cold shocks, osmotic and oxidative stresses, heavy metal exposure, irradiation, viral infection and high population densities. Hsps function as molecular chaperones that bind to partially denatured proteins, promote their correct refolding, translocation across membranes and prevent their aggregation. Although it has been well established that Hsps are participating in heat shock response, the mechanisms of cold injury and its reparation are not well understood in insects. In this report, we try to compare differences in *hsp70* expression profiles in the red fire-bugs (*Pyrrhocoris apterus; Heteroptera*) exposed to high and low temperatures, and use RNAi technique to study the role of *hsp70* gene transcriptional upregulation during the reparation of injury caused by the two thermal shocks.

## MATERIALS AND METHODS

**• *Pyrrhocoris apterus* and rearing conditions**

We tested the responses to **cold (-5°C/4d)** or **heat (+45.3°C/1h)** stimulus in the adults of *Pyrrhocoris apterus* (only males were used) that were previously reared in laboratory conditions of constant temperature of 25°C and a long-day photoperiod (LD) of 18h light:6h dark.

**• RNA extraction, cloning and sequencing of *hsp70* and *hsc70* genes**

Abdominal fat bodies were dissected and total RNA was extracted. cDNAs coding for *hsp70* and *hsc70* genes were obtained by the standard RT-PCR method using degenerated primers. PCR cDNA products were cloned into the pGEM-TEasy vector. The positive clones were cultured, their plasmid DNA extracted and sequenced on ABI Prism 377 DNA Sequencer.

**• qRT-PCR analysis**

Relative abundances of mRNA transcripts of *hsp70* and *hsc70* were measured by quantitative real-time PCR technique using Rotor Gene RG 3000 PCR Light Cycler. The abundance of mRNA transcripts of *rp49* gene was used as standard.

**• Downregulation of gene transcription by RNAi technique**

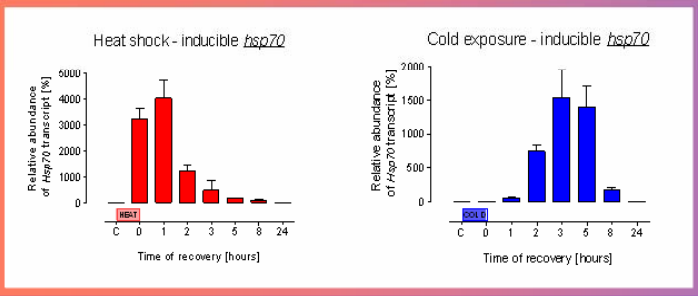
dsRNAs were prepared by in vitro transcription using the Ambion MEGAscript™ T7 Kit. After the injection of dsRNAs, individuals were kept under LD/25°C for 4 days prior to their exposure to either heat or cold.

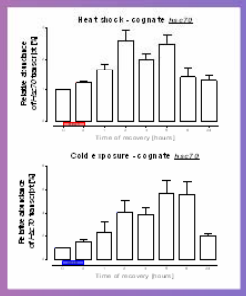
## RESULTS

### 1. Fragments of nucleotide sequences of structural homologs of inducible *hsp70* and cognate *hsc70* genes in the heteropteran *Pyrrhocoris apterus* were obtained.

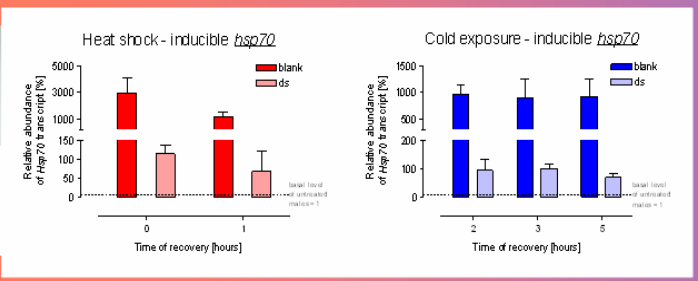
The 1 460 bp and 1 580 bp-long fragments of *hsp70* and *hsc70*, respectively, were sequenced and translated into the amino acid (aa) sequence. Comparing the aa-translation with known sequences in *Drosophila melanogaster* and in *Bemisia tabaci* revealed that 87% and 93% of the aa, respectively, are identical. *Hsp70* and *hsc70* evidently belong to the Hsp70 family.

### 2. The abundance of mRNA transcript for inducible *hsp70* gene increased by about three orders (1 000 – 4 000-fold) after the heat or cold shock. Expression of cognate *hsc70* gene increased up to 6-fold.

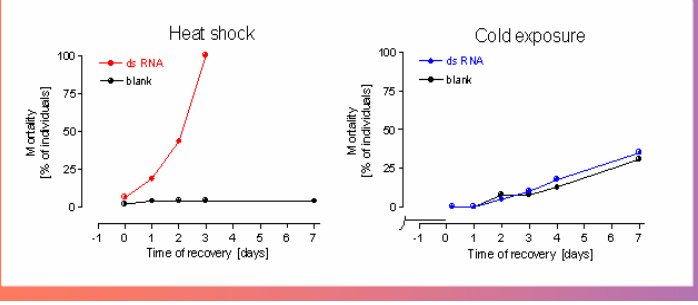



### 3. The injection of *hsp70* dsRNA suppressed the expression of *hsp70* gene in heat-shocked and cold-exposed insects.


### 4. Suppression of *hsp70* expression prevented reparation of heat-injury. In contrast, no effect of *hsp70* dsRNA treatment on reparation of cold-injury was observed.




## CONCLUSIONS

• Levels of inducible *hsp70* mRNA transcripts increased by about three orders after both heat-shock and cold-exposure. Levels of cognate *hsc70* mRNA increased only by about 6-fold.

• Injection of *hsp70* dsRNA resulted in a deep (but not complete) suppression of *hsp70* expression induced by both temperature treatments.

• While all heat-shocked insects that were injected by injection buffer (blank) successfully repaired heat injury and survived, those injected with *hsp70* dsRNA all died. It shows clearly that Hsp70 protein plays a vital role during heat-injury reparation.

• RNAi suppression of *hsp70* expression had no effect on recovery after the cold-exposure. It seems that either Hsp70 protein does not play any role in this process or our RNAi suppression was not sufficient to reveal its role.

## ACKNOWLEDGEMENT

This work was supported by the Czech Science Foundation (Grant No. 206/07/0269).

# ABSTRACT 4

**Expression of 70 kDa heat shock proteins in the bug, *Pyrrhocoris apterus*: What is their role in cold tolerance?**

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The heat shock proteins (Hsps) were originally discovered as being induced by heat shock. Later, it was found that a wide array of environmental stresses, including low temperatures, elicit a similar response. The up-regulation of Hsp mRNA levels in response to cold, or during overwintering diapause, was reported in many insects. Less often, such observations were extended to the protein levels and only seldom, the causality of the relationship between the up-regulation of Hsps and cold tolerance was directly tested and proved.

We will report on assessing the competence of the adults of *Pyrrhocoris apterus* (Heteroptera: Pyrrhocoridae) for responding to heat- and cold-stresses by up-regulation of 70 kDa Hsps and the role of these Hsps during repair of heat- and cold-induced injury. We found that the abundances of mRNA transcripts for two protein forms, inducible (PaHsp70) and cognate (PaHsc70), are significantly up-regulated in response to high and low temperature stimuli. At the protein level, only the inducible form showed a clear up-regulation response. Injection of 695bp-long *Pahsp70* dsRNA (RNAi) caused drastic suppression of the heat- and cold-stress-induced *Pahsp70* mRNA response and the up-regulation of corresponding protein was practically eliminated. Our RNAi predictably prevented recovery from heat shock and, in addition, negatively influenced repair of chilling injuries caused by cold stress. Although our results do support the hypothesis on active participation of heat shock proteins in the insect cold tolerance, we will also present some data, which stress the pitfalls of over-generalization.

3rd International Symposium on the Environmental Physiology of Ectotherms and Plants, Tsukuba, Japan, August 24-28, 2009.

J Comp Physiol B (2009) 179:569–577
DOI 10.1007/s00360-009-0341-9

ORIGINAL PAPER

# Seasonal changes in lipid composition and glycogen storage associated with freeze-tolerance of the earthworm, *Dendrobaena octaedra*

**Johannes Overgaard · Michaela Tollarova · Katarina Hedlund · Søren O. Petersen · Martin Holmstrup**

Received: 9 December 2008 / Revised: 2 January 2009 / Accepted: 6 January 2009 / Published online: 24 January 2009


**Abstract** The earthworm, *Dendrobaena octaedra*, is a common species in the uppermost soil and humus layers of coniferous forests and tundra in temperate and subarctic regions. The species is freeze-tolerant and may survive several months in a frozen state. Upon freezing, glycogen reserves are rapidly converted to glucose serving as a cryoprotectant and fuel for metabolism. In the present study we investigated the induction of freeze-tolerance under field conditions, and sought to find relationships between temperature, glycogen and fat reserves, membrane phospholipid composition and the degree of freeze-tolerance. Freeze-tolerance was induced when worms had experienced temperatures below 5°C for 2 weeks or more. Freeze-tolerance was linked to the magnitude of glycogen reserves, which also fluctuated with field temperatures being highest in autumn and winter. On the other hand fat reserves seemed not to be linked with freeze-tolerance at all. However, high glycogen alone did not confer freeze-tolerance; alterations in the membrane phospholipid fatty acid composition (PLFA) were also necessary in order to secure freeze-tolerance. The changes in PLFA composition were generally similar to changes occurring in other ectothermic animals during winter acclimation with an increased degree of unsaturation of the PLFAs.

**Keywords** Earthworms · Freeze-tolerance · Glycogen · Membrane lipids · Winter temperatures

Communicated by I.D. Hume.

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