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

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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.

INTRODUCTION

Pervasive effects of low temperature on ectotherm physiology have had a deep influence on evolution of insect overwintering strategies in temperate habitats. Insects may survive subzero temperatures by two general strategies which are most often referred to as "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 the supercooling point (Bale, 1993; Renault et al., 2002). Freeze-tolerant species survive freezing of their body fluids provided it is restricted to extracellular compartments (Lee & Denlinger, 1991; Sinclair et al., 2003). Many insects accumulate low molecular weight sugars and polyols during overwintering. They function as either colligative or non-colligative cryoprotectants, improve cold tolerance and enhance survival during unfavourable winter conditions (Zachariassen, 1985; Storey & Storey, 1991). Accumulated cryoprotectants cause colligative depression of melting and supercooling points in freeze-susceptible insects, regulate the minimum cell volume during dehydration caused by extracellular ice formation in freeze-tolerant species and protect functional structures of biological membranes and proteins (Crowe et al., 1987; Carpenter & Crowe, 1988; Storey & Storey, 1988, 1991). Glycogen reserves in the fat body serve as the main source for polyol biosynthesis (Hayakawa & Chino, 1981; Storey & Storey, 1981; Koštál et al., 2004a). Regulation of glycogen phosphorylase (GPase) activity most probably exerts the primary control over the activation of polyol synthesis (Ziegler et al., 1979; Hayakawa, 1985). GPase catalyses glycogenolysis which may increase the flow of carbon to the hexose monophosphate shunt and to metabolic pathways where sugars and polyols are synthesized (Storey & Storey, 1981). Low temperature is the immediate stimulus that initiates polyol accumulation in most insects (Ziegler et al., 1979; Storey & Storey, 1983). Nevertheless, other environmental cues are also involved, for instance photoperiod, food and water availability (Furusawa et al., 1982; Storey & Storey, 1986; Rojas et al., 1986; Hodková & Hodek, 2004).

The linden bug, *Pyrrhocoris apterus* L. (Heteroptera: Pyrrhocoridae), has been used as a model to investigate physiological and biochemical aspects of diapause and cold hardiness (Sláma, 1964; Hodek, 1968; Koštál & Šimek, 2000; Koštál et al., 2004a, b; Hodková et al., 1999, 2002). Brachypterous wing-morph adults enter a facultative reproductive diapause in response to subcritically short day-length (< 16 h 30 min) during the second half of summer (Hodek, 1968). During the warm period of autumn the bugs maintain their diapause and prepare for overwintering. Overwintering bugs remain inactive in the upper litter layer, supercool down to ca –20°C and do not tolerate freezing of their body fluids (Hodková & Hodek, 1997; Koštál & Šimek, 2000). During the cold period of autumn they gradually terminate diapause and diapause is completed after the winter solstice (Hodek, 1971). During cold winter months adults persist in a state of low temperature quiescence. When they are exposed to temperatures below 5°C they start to accumulate four specific polyols (ribitol, sorbitol, arabinitol and mannitol) which function as non-colligative cryoprotectants (Koštál et al., 2001). The ability to accumulate winter polyols is restricted only to the adults that have previously entered diapause. No polyol accumulation was detected in non-diapause reproducing adults (Šlachta et al., 2002).

The main aim of this study was to investigate seasonal changes in the activities of three enzymes involved in polyol synthesis (aldose reductase, AR; ketose reductase, KR; and polyol dehydrogenase, PDH) in adult females collected from the field. This way, our earlier laboratory results (Koštál et al., 2004 a, b) would be extended and verified. The study focused on the difference between reproductively arrested (either diapausing or quiescent) and reproductively active females (either overwintered or spring generation). Further, we tried to identify and describe seasonal trends in enzyme activity and relate this to

TABLE 1. Collection dates and developmental state of *Pyrrhocoris apterus* females used for analyses.

Collect. date	Developmental state of *P. apterus*
13 Sep. 2005	G0, diapause initiation *
25 Oct. 2005	G0, diapause maintenance
16 Nov. 2005	G0, diapause termination
26 Dec. 2005	G0, diapause termination/post-diapause quiescence
26 Jan. 2006	G0, post-diapause quiescence
23 Feb. 2006	G0, post-diapause quiescence
24 Mar. 2006	G0, post-diapause quiescence
24 Apr. 2006	G0, post-diapause, reproductive
15 May 2006	G0, post-diapause, reproductive
13 Jun. 2006	G1, non-diapause, reproductive *

* 5th instar larvae were collected on 1 Sep. 2005 and 1 Jun. 2006 and kept in an outdoor cage until the adults moult; ca. 1 week-old adults were then sampled. G0 – overwintering generation; G1 – first spring generation.

diapause development and to changes in ambient temperature. Post-diapause quiescent bugs collected in February were exposed to high temperatures (de-acclimated) and subsequently to low temperatures (re-acclimated) in laboratory, thereby testing their capacity to re-establish high activities of the three enzymes in response to cold stimulus.

MATERIAL AND METHODS

Insects, experimental conditions and sampling

Pyrrhocoris apterus bugs were collected regularly (approximately once a month) from September 2005 to June 2006 in Stromovka, České Budějovice (South Bohemia, Czech Republic). Only brachypterous females were used for the analyses. Collection dates and a basic description of the developmental state of the bugs are shown in Table 1. The meteorological data (daily temperature maxima and minima) for the cold season were provided by the weather station of the Czech Institute of Hydrometeorology in České Budějovice (Fig. 1).

Some females collected in February were de-acclimated in the laboratory, i.e. they were kept for 2 weeks under conditions which promote resumption of direct development (constant 25°C and long-day photoperiod of 18L : 6D) and the sample of reproductive females was taken. After this treatment, the bugs were subjected to gradual re-acclimation, i.e. alternating temperatures were applied during 3 weeks (thermophase/cryophase: 20°/10°C, 15°/5°C and 10°/0°C respectively). The insects were subsequently kept at constant 0°C and darkness for 1 week and sampled afterwards.

Enzyme activities

Methods previously described in Koštál et al. (2004a, b) were used. Briefly, three to ten independent samples were prepared at each sampling date. Each sample contained pooled abdominal fat bodies dissected from between five to eight females. Samples were homogenized in a buffer consisting of 100 mM Tris-HCl, pH 8.0, 15 mM mercaptoethanol and 1 mM EDTA. Supernatants obtained after centrifugation at 22,000 g for 20 min at 4°C were used as the source of the enzymes. Total protein concentrations in the enzyme preparations were measured by BCA protein assay (Stoscheck, 1990) and enzymatic activities were expressed as mmoles of substrate converted to product per min per g of total protein. The final activity values were calculated after subtracting blank values. Activities were measured at 25°C using Pye Unicam SP8-100 spectrophotometer (Cambridge, U.K.) by continuous time scanning at 340 nm. All chemicals were purchased from Sigma-Aldrich Co. (St. Louis, Missouri). Reaction mixtures consisted of 20 mM imidazole-HCl buffer (pH 7.2); and substrates: either 0.1 mM NADPH; 250 mM D-ribose (AR); 250 mM D-fructose (KR); or 0.15 mM NADH; 250 mM D-fructose (PDH).

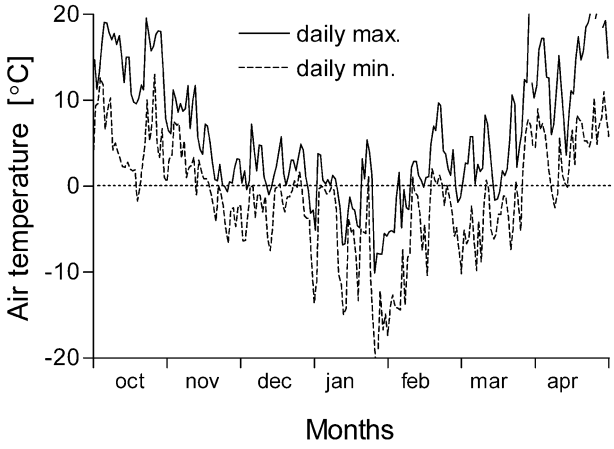

Fig. 1. Daily temperature maxima and minima from October 2005 to April 2006 provided by the weather station of the Czech Institute of Hydrometeorology in České Budějovice.

Statistical analysis

Differences between means (sampling dates) within a single (overwintering) generation were tested statistically by Anova followed by Tukey's multiple comparison test. The differences between reproductive females of two different generations, i.e. G0 (ovewintering generation, post-diapause females collected in May) and G1 (first spring genaration of females colected in June) were statistically analyzed using an unpaired t-test.

RESULTS AND DISCUSSION

Results are summarized in Fig. 2. Solid connecting lines represent the changes in enzyme activities of field collected insects during the season 2005/2006. Due to considerable individual variation no statistically significant differences were seen. Nonetheless clear trends were observed. The seasonal AR and KR trends were similar (Fig. 2a, b). Activities of AR and KR were relatively low during diapause initiation, slightly increased and stayed constant during diapause development and thereafter further increased to highest values with the transition to post-diapause quiescence during the winter months. A significant drop of activities was observed during the spring rise in temperature when the females resumed reproduction. The reproductive (G0) females collected in April showed slightly higher activities in comparison to females of the same generation collected in May. No difference was found between reproductive females of G0 (May) and G1 (June). Seasonal changes in PDH activity (Fig. 2 C) differred from those of AR and KR. Relatively high, but fluctuating, activities were seen from diapause initiation until the transition to low temperature quiescence. A gradual decrease in activity was apparent in post-diapause females during their low-temperature quiescence and in those females which resumed their development during spring.

Field data obtained in this study develop our earlier laboratory results (Koštál et al., 2004a, b). First of all, a basic difference between reproductively active (low activities of AR, KR, PDH) and arrested (high activities) females has been confirmed. Such a difference is in agreement with the fact that the capacity for polyol accumulation develops only in diapause females (Šlachta et al., 2002). Overall seasonal change profiles are, however, difficult to compare between field and laboratory where envi-

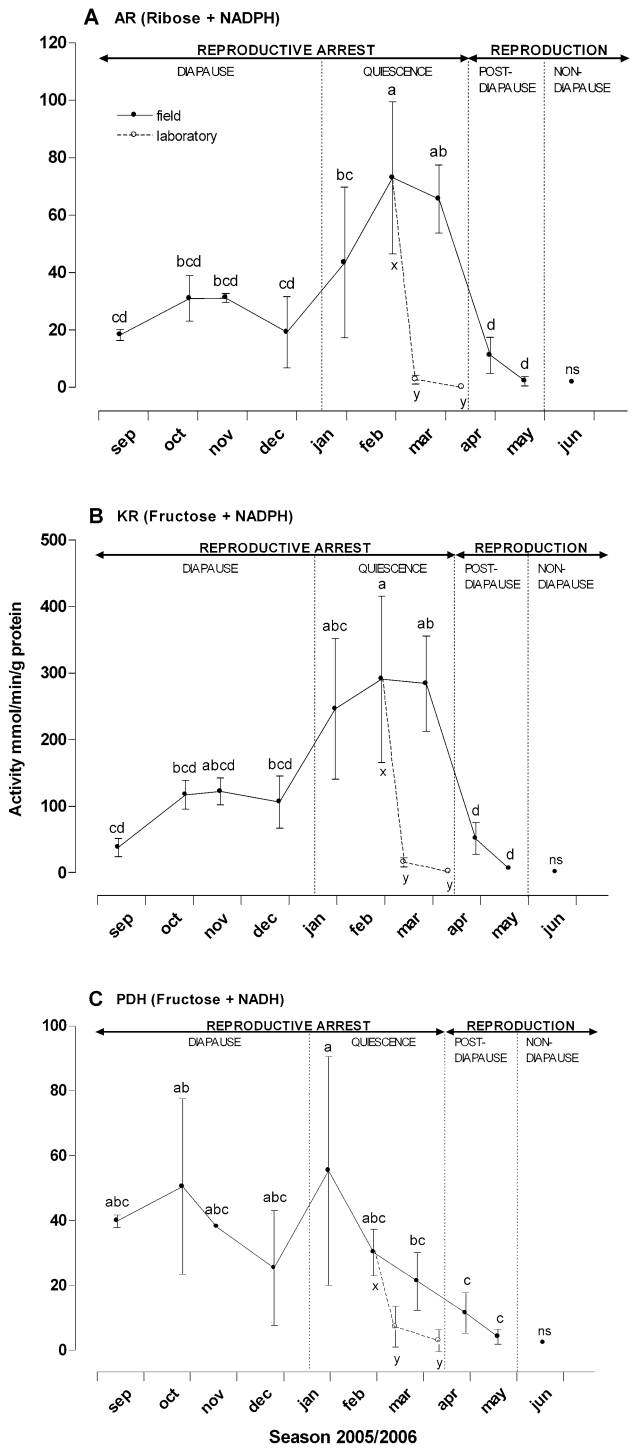

Fig. 2. Seasonal changes of activities of (A) aldose reductase (substrates: ribose, NADPH), (B) ketose reductase (substrates: fructose, NADPH), and (C) polyol dehydrogenase (substrates: fructose, NADH) in *Pyrrhocoris apterus* adult females collected from the field during 2005/2006 (full points, solid line) and after de-acclimation/re-acclimation in laboratory (empty points, dashed line). Each data point represents a mean ± SD (n = 3–10 samples, 5–8 female abdominal fat bodies in each sample). Means labeled by different letters were significantly different (ANOVA, followed by Tukey test, P = 0.05). The differences between post-diapause (G0, May) and non-diapause (G1, June) generations were not statistically significant (ns, $P > 0.05$).

ronmental conditions differed dramatically. Similarly, the relationships between enzyme activities and polyol accumulation were not straightforward. While ribitol accumulated throughout the cold season to similar concentrations, accumulation of sorbitol was much higher in the field during autumn (Koštál & Šimek, 2000; Koštál & Šlachta, 2001) or during early phases of diapause development in the laboratory (Koštál et al., 2004a). It has been shown that in many insects, including *P. apterus,* a drop in temperature below a certain threshold (most often around 5°C) is needed to stimulate rapid polyol accumulation (Ziegler et al., 1979; Storey & Storey, 1983; Koštál et al., 2001, 2004b). Such a threshold was reached in the field in approximately mid-November 2005. Nonetheless no increase in enzyme activities was seen between mid-November and late December when the air temperatures oscillated closely around 0°C. In two other insects, for which similar data are available, the correlations between seasonal changes in enzyme activities and polyol accumulation were more direct. Activity profiles of enzymes involved in glycerol synthesis corresponded well to the winter glycerol profile of *Chilo supressalis* (Li et al., 2002). Similarly, changes in the activity of aldose reductase (substrates: glucose and NADPH) paralleled the profile of sorbitol synthesis almost exactly during overwintering in *Eurosta solidaginis* (Joanisse & Storey, 1994).

In addition to environmental regulation by temperature changes, the enzyme activities might be under the influence of developmental signals. The termination of diapause and transition into a state of low-temperature quiescence during early winter coincided in time with the apparent increase of AR and KR activities which was countered by a decrease in PDH activity. Similar results were obtained in overwintering larvae of *Chilo suppresalis.* Both, the decreasing ambient temperatures and the transition from diapause to quiescence were probably responsible for changes in activities of enzymes associated with glycerol synthesis (Li et al., 2002).

Dashed lines in Fig. 2 represent the enzyme activities after laboratory de-acclimation, followed by gradual cold re-acclimation of quiescent females collected in February. The activities of all enzymes considerably decreased during de-acclimation and remained low during the subsequent attempt to re-acclimate them. Similarly, polyols were cleared during the de-acclimation and no re-accumulation was observed (unpublished data). Such a close correlation further supports the existence of a causal relationship between diapause, the high activities of AR, KR and PDH enzymes and the capacity to accumulate polyols. Once diapause has been terminated and direct development resumed, the capacity to accumulate polyols in response to low temperature stimulus was lost.

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