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EFFECTS OF TEMPERATURE AND PHOTOPERIOD ON GLUCOSE, GLYCEROL AND GLYCOGEN CONCENTRATIONS IN *HELIX POMATIA* LINNAEUS, 1758 IN SPRING AND AUTUMN

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ABSTRACT: One of the variables oscillating seasonally in *Helix pomatia* L., described in our previous paper, was their rudimentary cryoprotection provided by modest increases in haemolymph glucose and glycerol concentrations in autumn and early spring, respectively. Because factors governing cryoprotective responses are unknown, we decided to investigate the effects of spring and autumn acclimation of *H. pomatia* to different thermal and photoperiod conditions on the changes in haemolymph concentrations of glucose and glycerol and on the glycogen level in selected organs. Neither acclimation to short-day photoperiod nor low ambient temperature evoked increase in glucose and glycerol concentration in spring and autumn. Both acclimation variants decreased hepatopancreatic glycogen level. The rudimentary freeze-tolerance in *H. pomatia* seems to be a combined effect of cold and short-day photoperiod and might also be affected by their nutritional and reproductive status. The effect of exposure to frost-bite is also likely to be involved.

KEY WORDS: overwintering, freeze-tolerance, acclimation, cryoprotectants, land snails, *Helix pomatia*

INTRODUCTION

Entering winter torpor in land snails is frequently related to decrease in ambient temperature (LAZARIDOU-DIMITRIADOU & SAUNDERS 1986), shortening of light phase, and low air humidity during autumn. However, before the onset of winter some preparatory changes, such as local migration, burrowing underground, calcareous epiphragm formation, decrease in body water content (BIANNIC & DAGUZAN 1993, BIANNIC et al. 1994), and changes in activity of some enzymes (BIELAWSKI & KĘSA 1986) have been reported. Despite the decreased ambient temperature in winter, torpid land snails are able to increase activities of their glycolytic enzymes (MICHAELIDIS et al. 2008).

In the temperate zone, where the ambient temperature drops below the freezing point in winter, ectothermic animals use two anti-freezing strategies: (i) freeze-tolerance – using low-molecular-weight organic solutions as cryoprotectants and (ii) freeze-avoidance – enhancing the ability of body fluids to supercool (STOREY & STOREY 1988). Freeze-tolerance depends on accumulation of polyols and sugars which prevent intracellular ice formation. Synthesis of cryoprotectants prior to entering winter torpor is well known in some invertebrates (LI et al. 2002) and vertebrates (LAYNE 1995). Elevated synthesis of these substances is associated with hormonal changes in insects (WATANABE & TANAKA 1999), with seasonal changes in lizards (GRENOT et al. 2000, VOITURON et al. 2000, 2002) and is affected by extracellular ice formation in frogs (STOREY & STOREY 1985, STOREY 1987).

According to some authors freeze tolerance in land snails depends on their size (ANSART & VERNON 2004), emptying of their gut (ANSART et al. 2002) and decreasing their body water content (NICOLAI et al. 2005). Seasonal changes in the haemolymph concentration of cryoprotectants, such as glycerol and glucose, in *H. pomatia* Linnaeus, 1758 do not depend on organ dehydration (NOWAKOWSKA et al. 2006). There

is a modest increase in glucose concentration in winter. On the other hand, glycerol concentration is slightly elevated in spring-active snails, which are well hydrated but frequently exposed to freezing during episodes of night ground frosts.

The cellular mechanisms responsible for regulation of freeze tolerance in land snails are still far from being clear. Therefore, the objective of this study was to verify the exogenous/endogenous control of cryoprotectants synthesis in *H. pomatia*. The first aim of the present study was to record changes in the haemolymph concentrations of glucose and glycerol in snails acclimated to different thermal and photoperiod conditions during spring and autumn. We also decided to determine glycogen concentration in selected organs because glucose and glycerol synthesis is based on catabolism of liver glycogen (STOREY et al. 1981, STEINER et al. 2000, LI et al. 2002). Another purpose of our study was to determine whether seasonal changes in the cryoprotectant concentration in *H. pomatia* resulted from internal clock or were a response to environmental changes. We applied acclimation to various combinations of ambient temperature and photoperiod to analyse various aspects of intermediate metabolism, including synthesis of glucose and glycerol. Accordingly, we checked separately the effect of temperature and photoperiod on the biochemical changes, which could occur over time preceding or following torpor in nature.

MATERIAL AND METHODS

ANIMALS

A total of 38 adult *H. pomatia*, weighing approximately 24 g, were collected from their natural habitat (permission of the Polish Ministry of Environmental Protection No.WsiR.II.KLD-6631-209/05) in the vicinity of Toruń (central Poland, 53°02'N, 18°35'E), twice over a period of their natural activity (i) in spring (April), just after their arousal from winter torpor and (ii) in autumn (October), prior to natural onset of winter torpor. All the snails were weighted and the height of their shells was measured. Only adult individuals with developed lip were used.

EXPERIMENTS

The first set of experiments was performed in the spring. Immediately after bringing the snails to the laboratory, they were exposed to (i) summer-specific ambient temperature of 25°C combined with short-day photoperiod (8L:16D) and (ii) summer temperature (25°C) combined with long-day photoperiod (16L:8D), for three weeks preceding biochemical analyses. Spring-active control snails were taken from the field at the end of the acclimation period in order to reduce the effect of season on cryoprotectants concentration, which had been shown previously (NOWAKOWSKA et al. 2006). When the snails were collected, the ambient temperature was 8°C and the photoperiod was 15L:9D. Data concerning summer-specific ambient conditions (temperature of 24°C and long-day photoperiod of 16L:8D) were taken from our previous investigation (NOWAKOWSKA et al. 2006).

The second set of experiments was performed in the autumn. The freshly collected snails were divided into three groups which, during three weeks, were subject to acclimation to the following photoperiod and temperature combinations: (i) summer-specific ambient conditions (25°C, 16L:8D), (ii) summer temperature (25°C) combined with short-day photoperiod (8L:16D) and (iii) autumn temperature (5°C) combined with long-day (16L:8D) photoperiod. Autumn control data were taken from our previous paper (NOWAKOWSKA et al. 2006). When the control snails were collected, the ambient temperature was 9°C and the photoperiod was 10L:14D.

During acclimation the snails were housed in large acrylic boxes of 0.4 × 0.3 × 0.2 m, covered with wire mesh, with leaf litter from their natural habitat. They were fed ad libitum with fresh lettuce and vegetables. Fragmented shells were provided as a calcium source.

In both series of experiments biochemical analyses were carried out after acclimation. The snails were used post mortem to examine haemolymph concentrations of glycerol and glucose as well as organ concentration of glycogen. They were decapitated, and haemolymph samples were taken by puncturing the heart with a syringe needle. The foot, kidney and hepatopancreas were removed and used to determine organ glycogen concentration.

LABORATORY ANALYSES

Chemicals: Anthrone reagent, potassium hydroxide (KOH), trichloroacetic acid (TCA), were purchased from Polskie Odczynniki Chemiczne (Gliwice, Poland) and o-toluidine was purchased from Sigma-Aldrich GmbH (Steinheim, Germany). All other reagents were of analytic grade. All solutions were prepared with deionized water.

Determination of glycerol concentration: The volume of haemolymph samples used to examine glycerol content was 0.1 ml. Haemolymph concentration of glycerol was tested enzymatically using commercial kits (Boehringer Mannheim Corp, Germany). Briefly, the hemolymph samples were diluted in 0.4 ml of

double-distilled water. The samples were incubated at 100°C for 5 min, then quickly cooled, and centrifuged at 8,000 g × for 5 min. Finally, 0.1 ml of the supernatant was used for the enzymatic reaction. The samples were subject to spectrophotometer (SEMCO S91E, Warsaw, Poland) analysis at 365 nm. Glycerol concentration was expressed in mmol/l.

Determination of glucose concentration: The haemolymph glucose concentration was assessed from reaction of o-toluidine with glucose, which is the main haemolymph monosaccharide in molluscs (BORGES et al. 2004). Glucose and o-toluidine give a colour complex. Briefly, 0.1 ml of the hemolymph was added to 1 ml of 3% TCA, and the samples were centrifuged at 10,000 g × for 5 min. Then, 0.2 ml of the supernatant was added to 1.8 ml of o-toluidine reagent and the samples were incubated in boiling water for 8 min. The absorbance of the colour complex was determined at 630 nm. Commercially available glucose (5.5 mmol/l) was used as a standard (Carmay, Lublin, Poland). Glucose concentration was expressed in mmol/l.

Determination of glycogen concentration: Glycogen concentrations in the kidney, hepatopancreas and foot were determined with anthrone method, previously described by SEIFTER et al. (1950) and modified by us. The shell of each snail was broken, its body was removed quickly and then foot, hepatopancreas, and kidney were extracted within 1 min. The organs were weighted using a precise (accuracy ±1 mg) balance (Axis, AD 300, Gdańsk, Poland) to determine their fresh mass and then they were put into flasks, and immediately dissolved in 3 ml of KOH per 1 g of fresh mass. The samples were heated in a boiling water bath for 20 min. The contents of the flasks were diluted in 50 ml of water. The water solution of each sample (1 ml) was poured into tubes and 5 ml of anthrone was carefully added. The tubes were then incubated at 100°C for 10 min. Following cooling, the samples were subject to spectrophotometer analysis at 620 nm. Glycogen concentrations were assessed using 5.5 mmol/l glucose standard (Carmay, Lublin, Poland) and expressed in μg/g tissue.

DATA ANALYSIS

The results were presented as mean values ±SE. Mean concentrations of cryoprotectant substances in the haemolymph were compared using variance analysis (one-way ANOVA), followed by the T-Tukey (Spjotvolla-Stoline'a test) post-hoc test. Two-way ANOVA was also used to analyse significance of organ changes in glycogen concentration and of the effect of acclimation. Differences between the groups were regarded as significant at $P<0.05$. Moreover, unpaired Student's t-test was used to compare spring and autumn changes of glucose and glycerol concentrations in snails acclimated to summer thermal conditions at long-day photoperiod with summer control data taken from our previous paper (NOWAKOWSKA et al. 2006).

RESULTS

EFFECTS OF TEMPERATURE AND PHOTOPERIOD ON CRYOPROTECTANT CONCENTRATIONS IN SPRING ACCLIMATION

Figure 1 shows that the spring acclimation to different environmental conditions did not induce changes in the haemolymph concentrations of glycerol (one-way ANOVA; $F_{(1,16)}=0.20$, n.s.) and glucose (one way ANOVA $F_{(1,15)}=0.33$, n.s.). Moreover, these concentrations were also unaffected by the shortening of the light phase ($F_{(1,9)}=0.76$, n.s.; $F_{(1,9)}=0.00$, n.s., respectively).

The glycogen concentration (Fig. 2) was much affected by the acclimation ($F_{(1,57)}=14.85$, $p<0.001$) and was organ-dependent ($F_{(2,57)}=21.86$, $p<0.001$); there was a significant interaction between these factors ($F_{(2,57)}=8$, $p<0.001$). The highest glycogen concentration was recorded in the hepatopancreas of spring-active control snails; it was significantly higher than that observed in the animals acclimated to long- ($p<0.05$) and short-day ($p<0.001$) photoperiod. In the kidney and foot of both acclimated groups the glycogen level was unaffected by the environmental conditions. However, the hepatopancreatic glycogen concentration in the snails acclimated to long-day photoperiod was higher ($p<0.001$) than in those acclimated to short-day photoperiod. In both control snails and those acclimated to long-day photoperiod the foot and kidney glycogen concentrations were highly significantly lower ($p<0.001$), compared to that in the hepatopancreas. Gradients of glycogen concentration between the organs during acclimation to the summer temperature of 25°C combined with short-day photoperiod were different from those recorded in either the spring control snails or those acclimated to the summer-specific ambient conditions (25°C, 16L:8D). In the former the highest concentration was recorded in the kidney, whereas in the latter – in the hepatopancreas.

The glucose concentration in the spring snails acclimated to the summer-specific ambient temperature of 25°C combined with long-day photoperiod (16L:8D) (Fig. 3A) was lower ($p<0.001$), compared to that in the summer control snails (data from our previous study; NOWAKOWSKA et al. 2006), but there was no difference

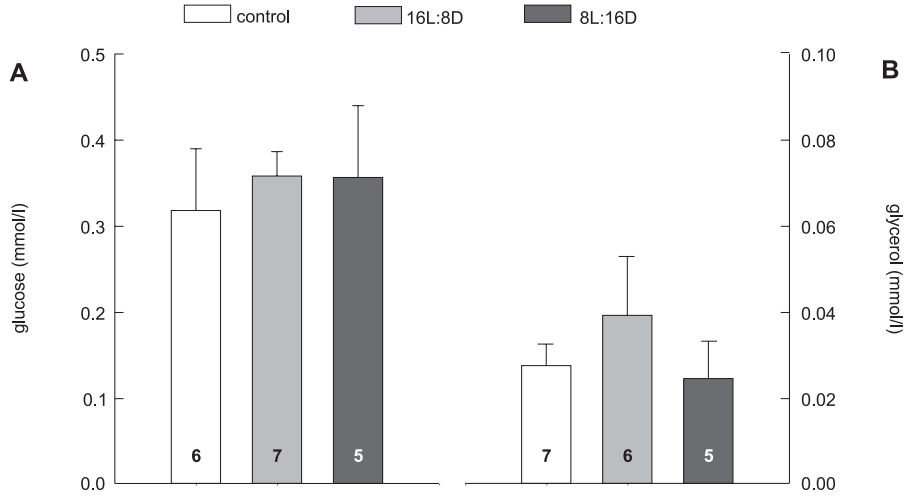

Fig 1. Effect of three-week acclimation to 25°C at a long-day (16L:8D) and a short-day (8L:16D) photoperiod on haemolymph glucose (panel A) and glycerol (panel B) concentrations in *H. pomatia* in spring. Control group – spring-active snails. Values given are means ±SE. Statistics: one-way ANOVA. Numbers inside columns indicate numbers of snails in individual groups

in the glycerol level (Fig. 3B) between those experimental groups.

The glycogen concentrations in the organs were affected by the spring acclimation to the summer specific environmental conditions (Fig. 3C). Two-way ANOVA showed a significant main effect of the type of organ ($F_{(2,54)}$=13.19, p<0.001) and an effect of season ($F_{(1,54)}$=6.98, p<0.01); there was a significant interaction between these factors ($F_{(2,54)}$=5.74, p<0.01). The highest concentration was recorded in the hepatopancreas of the spring snails acclimated to the summer-specific ambient conditions, and the post hoc test showed that it was significantly higher (p<0.01) than in the summer control snails. It was also significantly higher than in the kidney (p<0.001) and foot (p<0.001) of the acclimated snails.

EFFECTS OF TEMPERATURE AND PHOTOPERIOD ON CRYOPROTECTANT CONCENTRATIONS IN AUTUMN

Figure 4 shows that the autumn acclimation did not induce changes in the glucose concentration (one-way ANOVA, $F_{(1,33)}$=0.14; n.s.) but it strongly affected the glycerol concentration (one-way ANOVA, $F_{(1,37)}$=5.17; p<0.05). There were no differences in the glucose concentration between all acclimated groups, or compared to the autumn-active control snails (data from our previous study; NOWAKOWSKA et al. 2006). The highest glycerol concentration was recorded in the control snails and was significantly different (p<0.05) from those in each acclimation variant (post hoc Spjotvolla-Stoline's test). Further analysis showed

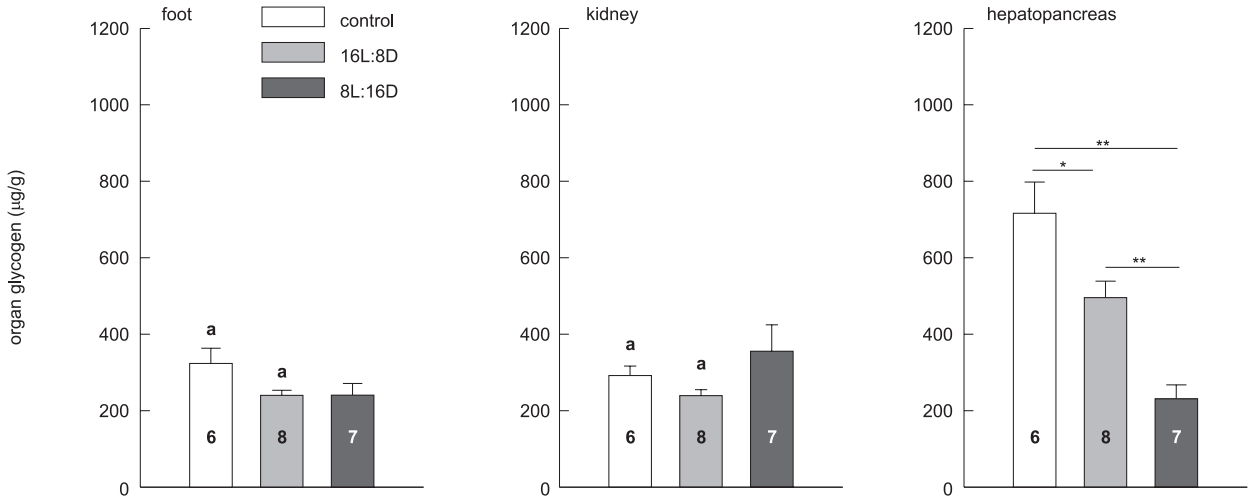

Fig. 2. Effect of three-week acclimation to 25°C at a long-day (16L:8D) and a short-day (8L:16D) photoperiod on glycogen concentrations in organs of *H. pomatia* in spring. Control group – spring-active snails. Values given are means ±SE. Statistics: two-way ANOVA followed by the Tukey post hoc test; asterisks indicate significant differences (*p<0.05, **p<0.001); a – significantly different from the values in the hepatopancreas in the corresponding groups (p<0.001). Numbers inside columns indicate numbers of snails in individual groups

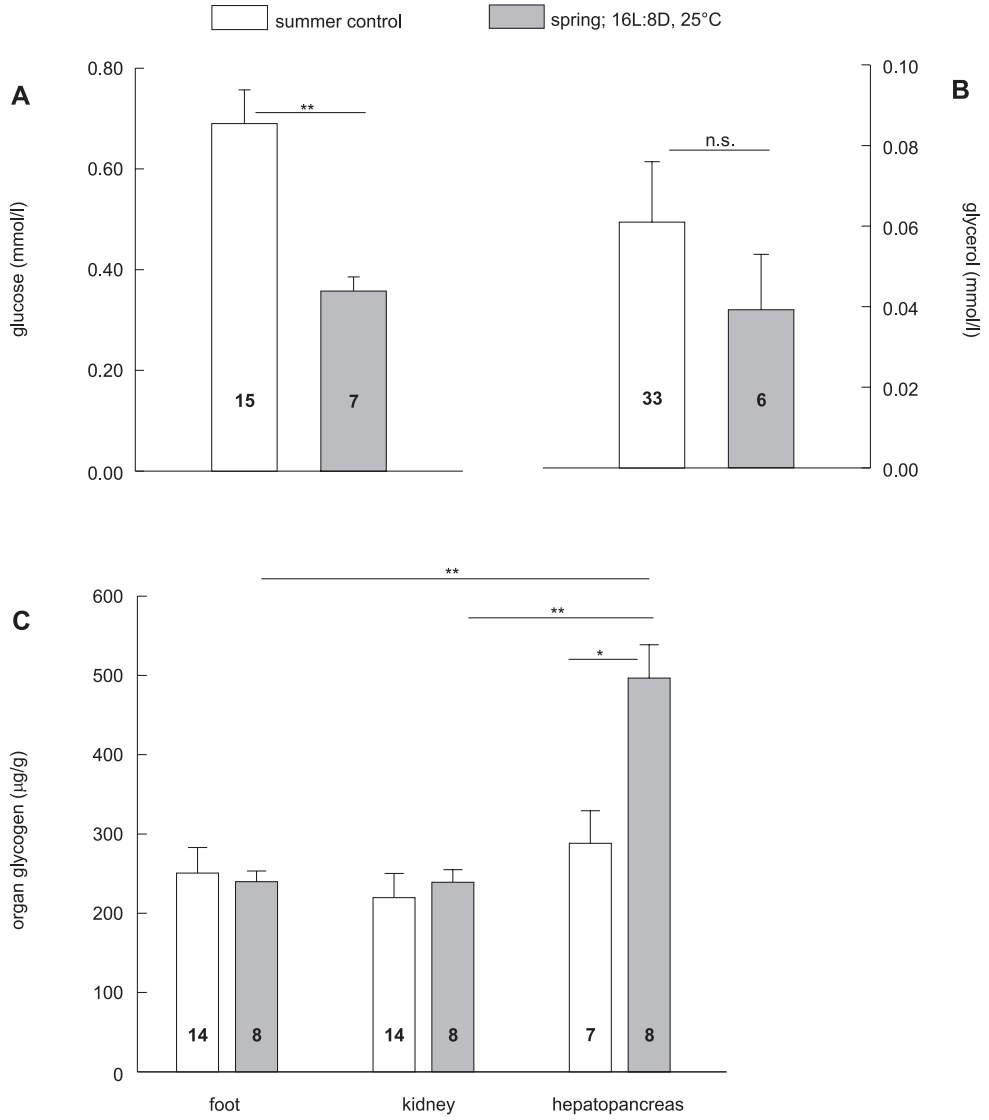

Fig. 3. Comparison of three-week acclimation to summer-specific ambient temperature of 25°C combined with long-day photoperiod (16L:8D) in spring with summer values recorded in our previous investigation (NOWAKOWSKA et al., 2006). Values given are means ±SE. Statistics: Student's t-test (panel A and B) and two-way ANOVA followed by the Tukey post hoc test (panel C); asterisks indicate significant differences (*p<0.01, **p<0.001, n.s. – not significant). Numbers inside columns indicate numbers of snails in individual groups

that there were no differences in the glycerol level between the acclimation variants.

The autumn experiments also showed that the glycogen concentration (Fig. 5) was strongly influenced by the acclimation ($F_{(1,72)}$=68.53; p<0.001) and by the type of organ ($F_{(2,72)}$=13.87; p<0.001) (two-way ANOVA). Moreover, a significant interaction between these factors was observed ($F_{(2,72)}$=3.19; p<0.05). The highest glycogen concentration was recorded in the hepatopancreas of autumn-active control snails and it was significantly higher, compared to that in the foot (p<0.01). In contrast, there were no differences in the organ glycogen concentration between the three acclimation variants.

The haemolymph concentrations of glucose (Fig. 6A) and glycerol (Fig. 6B) in the autumn snails acclimated to the summer-specific ambient conditions were not significantly different from those recorded in the summer control snails (data from our previous study; NOWAKOWSKA et al. 2006). The glycogen concentration (Fig. 6C), however, was organ-dependent ($F_{(2,46)}$=9.38; p<0.001); it was unaffected by the season ($F_{(1,46)}$=0.30; n.s.), but there was a significant interaction between these factors ($F_{(2,46)}$=3.77; p<0.05) (two-way ANOVA). The hepatopancreatic glycogen concentration in the snails acclimated to the summer-specific ambient temperature combined with short-day photoperiod was significantly higher than that recorded in the foot (p<0.01) and kidney (p<0.01), and than that recorded in the hepatopancreas of the summer control snails (p<0.05).

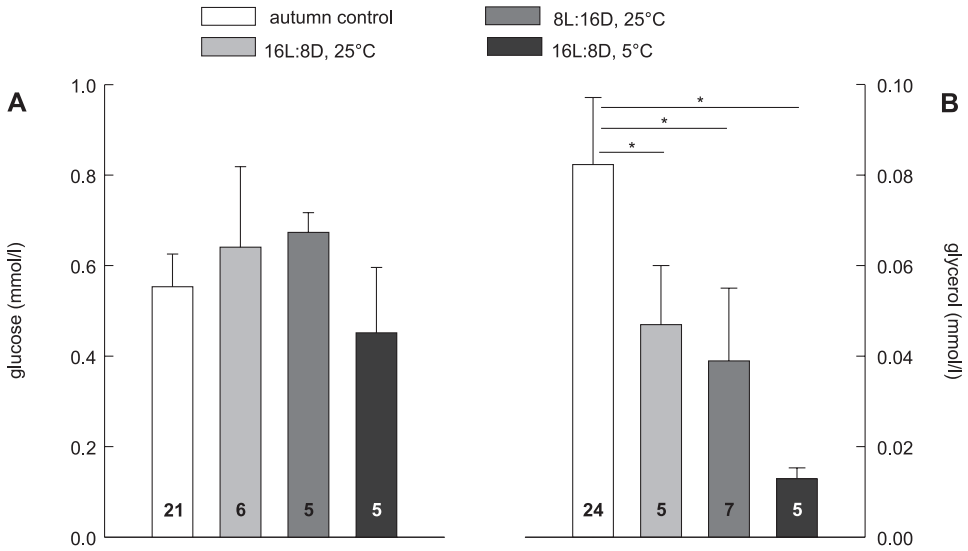

Fig. 4. Effect of three-week acclimation to 25°C at a long-day (16L:8D) and a short-day (8L:16D) photoperiod and to 5°C at a long-day photoperiod on hemolymph glucose (panel A) and glycerol (panel B) concentration in *H. pomatia* in autumn. Control data for autumn-active snails from NOWAKOWSKA et al. (2006). Values given are means ±SE. Statistics: one-way ANOVA followed by the Tukey post hoc test; asterisks indicate significant differences (*p<0.05). Numbers inside columns indicate numbers of snails in individual groups

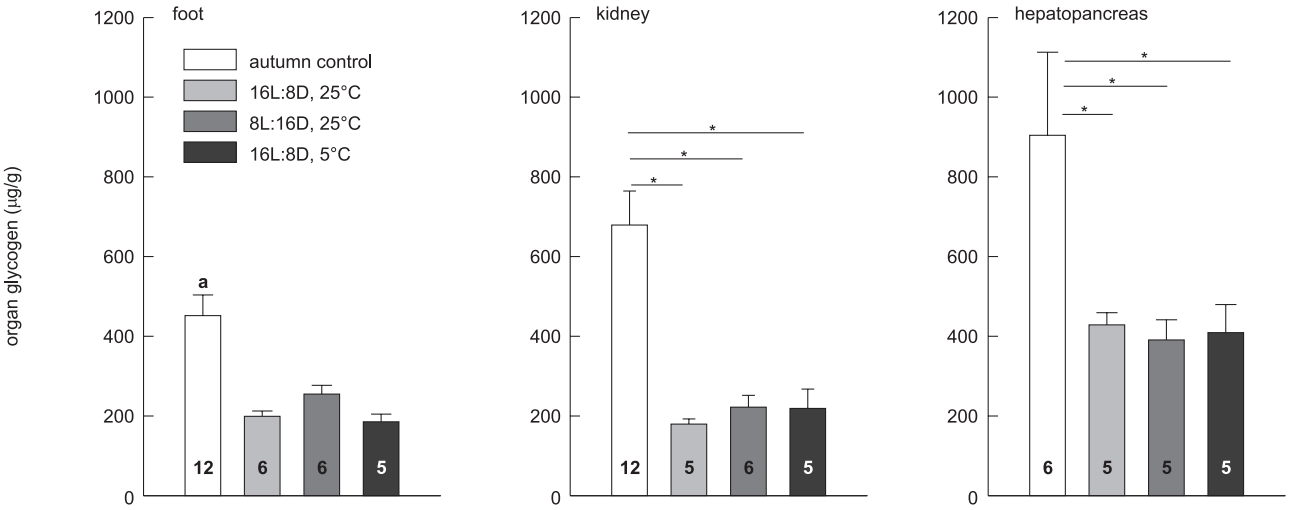

Fig. 5. Effect of three-week acclimation to 25°C at a long-day (16L:8D) and a short-day (8L:16D) photoperiod and to 5°C at a long-day photoperiod on organ glycogen concentrations in *H. pomatia* in autumn. Control data for autumn-active snails from NOWAKOWSKA et al. (2006). Values given are means ±SE. Statistics: two-way ANOVA followed by the Tukey post hoc test; asterisks indicate significant differences (*p<0.01), a – significantly different from the values in the hepatopancreas in the corresponding group (p<0.001). Numbers inside columns indicate numbers of snails in individual groups

DISCUSSION

Behavioural responses accompanying overwintering in molluscs are well understood. To avoid extreme cold exposure, terrestrial species shelter in buffered microhabitats, intertidal snails dig into the mud and freshwater species migrate to deeper water. Because terrestrial species of the temperate zone have to face huge variations in ambient temperatures (including temperatures markedly below 0°C), they must be able to prepare physiologically for the winter before the danger of freezing occurs.

There are two reasons to believe that winter torpor in *H. pomatia* is precisely regulated. Firstly, we have shown the endogenous control of arousal from winter torpor (CAPUTA et al. 2005). Secondly, we have recorded slight seasonal changes in the concentration of cryoprotectants, with the highest glucose level in the autumn (prior to epiphragm formation) and the highest glycerol concentration in the winter and spring (NOWAKOWSKA et al. 2006). The changes are too small to influence the freezing point of the snail's

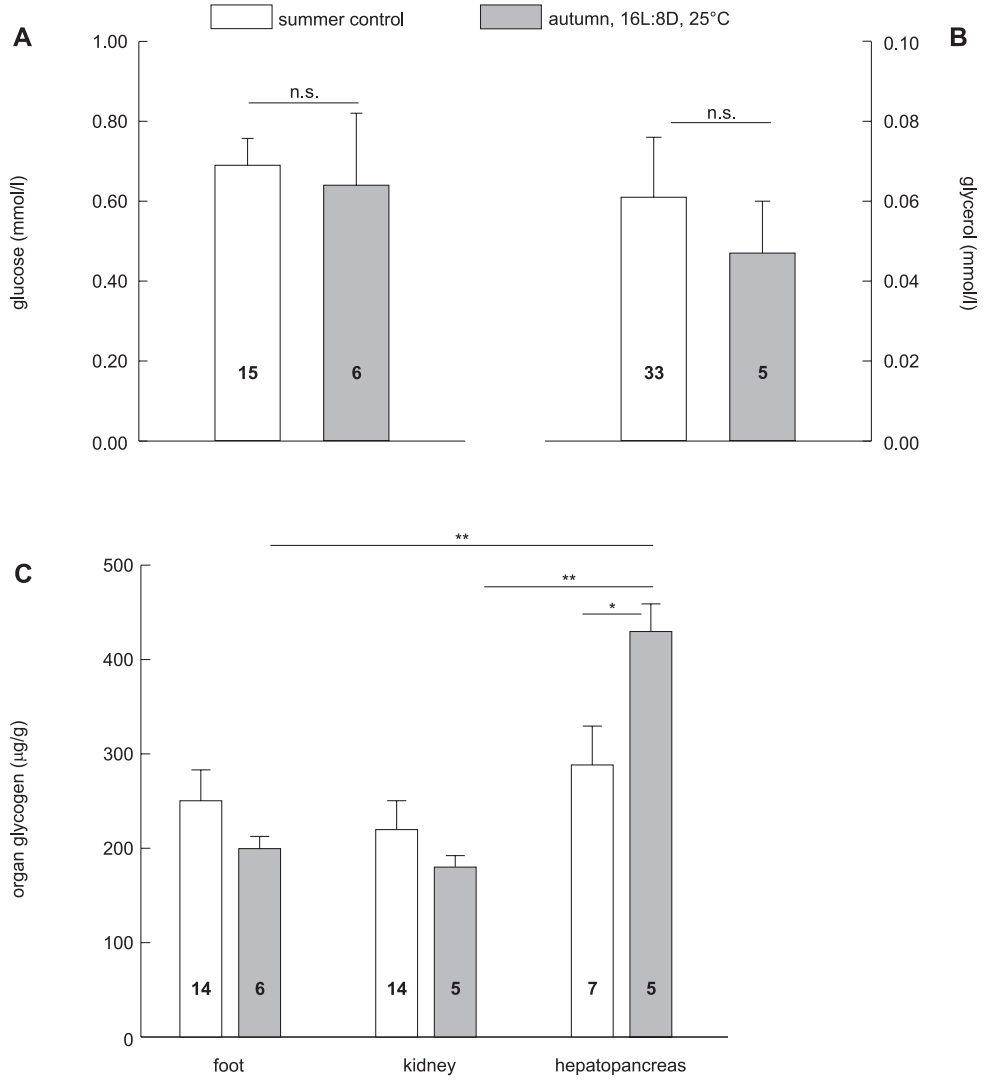

Fig. 6. Comparison of three-week acclimation to summer-specific environmental conditions in autumn with summer control values recorded in our previous investigation (NOWAKOWSKA et al. 2006). Values given are means ±SE. Statistics: Student's t-test (panel A and B) and two-way ANOVA followed by the Tukey post hoc test (panel C); asterisks indicate significant differences (*p<0.05, **p<0.01, n.s. – not significant). Numbers inside columns indicate numbers of snails in individual groups

body fluids. Therefore, they might be regarded as rudimentary cryoprotective seasonal responses.

The data obtained in this study show that the glucose and glycerol concentrations are unaffected by the spring acclimation to summer-specific thermal and photoperiod conditions. It must be emphasised that the control glycerol concentration in the spring in this study is not fully comparable with that from our previous paper (NOWAKOWSKA et al. 2006). Previously, we tested animals collected in the field immediately after their arousal from winter torpor and we found that they had then the highest glycerol concentration in their circannual cycle. In this study the control snails were taken from their natural habitat at the end of acclimation period, that is at least three weeks after their arousal from winter torpor. Apparently, by then the peak of glycerol concentration must have been over.

The differences in haemolymph glucose and in hepatopancreatic glycogen concentrations between the summer control data from our previous paper (NOWAKOWSKA et al. 2006), and those of the spring acclimation to summer-specific thermal and photoperiod conditions (see Fig. 3) are likely to be due to different nutritional conditions, but they might also reflect differences in the status of the snails' reproductive system (BRIDE et al. 1993). The elevated concentration of hepatopancreatic glycogen, accompanied by the reduced haemolymph glucose concentration, in the spring snails acclimated to summer-specific conditions can be regarded as a mobilisation of carbohydrates for the albumen gland.

The acclimation to summer thermal and/or photoperiod conditions in the autumn evoked decreases in the glycerol concentration in all the experimental groups of snails whereas the glucose level did not change, compared to that in the autumn-active snails. The former was rather surprising, especially in the light of BIANNIC & DAGUZAN's (1993) finding of a

predominant role of decreasing day length in the development of cold hardiness in *Helix aspersa*. In their study adult *H. aspersa* acclimated to a short-day photoperiod (12L:12D) had a greater supercooling ability than individuals acclimated to a long-day photoperiod (16L:8D) at 20°C. They suggested, however, that preparatory antifreezing responses were not associated with accumulation of cryoprotectants. Similarly, RIDDLE (1981) noticed that cold hardiness in *Anguispira alternata* was not associated with accumulation of a polyhydric alcohol and AARSET (1982) demonstrated that overwintering ability in *Littorina littorea* was independent of acclimation temperatures and photoperiod but shifted according to an endogenous circannual rhythm.

The reduced glycerol concentration, recorded in our acclimated snails in the autumn (see Fig. 4B), may also mean that not only low ambient temperature and short photoperiod but also other environmental conditions trigger entering torpor by the snails. It is also possible that the temperature of 25°C combined with the short-day photoperiod, applied in this investigation, was too high to activate preparatory responses preceding the torpor. However, acclimation to a short-day photoperiod at a low ambient temperature (5°C) in the summer leads to an elevated glycerol concentration (NOWAKOWSKA et al. 2006), but a prolonged acclimation (8 weeks) reverses this response, leading to a reduced cryprotectant concentration. Thus it cannot be excluded that a cue other than temperature and photoperiod is involved in initiation of glycerol synthesis in *H. pomatia*. Their reproductive cycle might be one of the factors. This conjecture is supported by the existence of a relationship between carbohydrate metabolism and function of female accessory sex glands in *H. aspersa* (BRIDE et al. 1993)

Acclimation experiments, performed in October, have shown that larvae of the gall fly *Eurosta solidaginis* accumulate glycerol in their haemolymph even at an ambient temperature of 15°C, well above the level at which cryoprotection would be necessary (STOREY et al. 1981). This suggests that changes in glycerol level depend upon the developmental stage. Because the body size affects cold hardiness in land snails (ANSART & VERNON 2004), in our experiments only adult snails with developed lip were used. HAN & BAUCE (1995) have also reported that overwintering in insects depends not only on temporal changes in temperature but on internal developmental processes and on interaction between them. In *H. pomatia* both decreasing ambient temperature and internal physiological state are likely to affect glucose and glycerol concentrations. This is confirmed by different patterns of changes in the cryoprotectants level in our snails acclimated in spring and autumn.

Glycerol synthesis in some invertebrates, such as insect larvae, is associated with changes in the activity of the respective enzymes (LI et al. 2002). Therefore, further studies are needed to investigate the role of enzyme activities in preparatory functions prior to winter torpor in snails. The activity of the enzymes seems to be correlated with breakdown of glycogen for glycerol synthesis (LI et al. 2002). There is a growing body of evidence pointing to a causal connection between synthesis of glucose and glycerol on the one hand, and glycogen catabolism on the other (STOREY et al. 1981, STEINER et al. 2000, LI et al. 2002). In our *H. pomatia* acclimated to different laboratory conditions there was no correlation between the changes in glucose and glycerol levels and glycogen concentration. Neither acclimation to short-day photoperiod at an ambient temperature of 25°C nor acclimation to long-day photoperiod at 5°C affected the glycogen concentration in their organs. In our previous paper (NOWAKOWSKA et al. 2006) we showed that in annual cycle of *H. pomatia* the hepatopancreas glycogen concentration was always higher than that in the kidney and foot. It means that the snail hepatopancreas is a glycogen reservoir, like in some vertebrates, but the correlation between glycerol and glucose synthesis and glycogen catabolism is absent. Because glycogen accumulation in hepatocytes of the turtle *Chrysemys picta* allows to bind a large amount of water inside the cells (STOREY 2006) the elevated glycogen concentration in the hepatopancreas of *H. pomatia* might play a role in their natural cryoprotection. Laboratory experiments at an ambient temperature of 25°C sometimes made snails estivate and, as a consequence, they starved for a couple of days. Both spring and autumn acclimation under such conditions is likely to induce decreases in hepatopancreatic glycogen level as a result of glycogenolysis to prevent drops in glucose level. Simultaneously, autumn production of glycerol in the acclimated snails must have been compromised by the partial depletion of the hepatopancreatic glycogen.

In conclusion, *H. pomatia* do not exhibit a clear-cut freeze tolerance. Seasonal changes in the cryoprotectants concentrations in their organs and simultaneous lack of clear evidence on endogenous control of their cold hardiness make this aspect of overwintering mechanism difficult to explain. Their rudimentary freeze-tolerance seems to be a combined effect of cold exposure and short-day photoperiod. The development of cryoprotection might also be dependent on exposure to a frost-bite. In that case, maintenance of slightly elevated glycerol concentration at the beginning of active season (NOWAKOWSKA et al. 2006) would enable *H. pomatia* to start the activity and reproduction early in the spring despite unfavourable thermal conditions.

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