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ARTICLE

Temporal dependence between hibernation and post-hibernation period according to biochemical profile of hemolymph in *Helix pomatia* Linnaeus, 1758

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ABSTRACT The first data regarding protein and mineral concentration during hibernation were obtained in the species *Helix pomatia*. Total proteins, albumins, globulins, Ca^{2+} and K^{+} concentrations were measured at the beginning of hibernation, end of hibernation and post-hibernation period (control group). Hemolymph was collected from the pericardial cavity. Total proteins were analyzed using the Biuret method, albumins were analyzed by BCG method, while calcium and potassium concentrations were obtained by CPC method and turbidimetric method, respectively. During hibernation the total protein, globulin and calcium levels were decreased, while the concentration of potassium was evidently increased. Total protein, globulin and calcium levels had the highest values in post-hibernation period. Exceptionally high values were obtained for calcium and globulin concentrations. Significant positive correlation between protein and globulin levels was established. Protein synthesis is the energy-utilizing process during metabolic depression and in our study decreasement of protein concentration in general has been shown during hibernation period. **Acta Biol Szeged 61(2):129-134 (2017)**

KEY WORDS

biochemical parameters
calcium ions
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snail

Introduction

In the animal world there are different strategies to survive extreme conditions, especially low temperatures. Survival is maintained at a low metabolic rate in order to reduce body temperature, mobility and other physiological functions at all levels of organization in various organisms due to different reasons (Ansart et al. 2002).

Decreased physiological activity also known as torpor is the main feature of hibernating animals which they use for surviving unfavorable seasonal climate changes and modified climate conditions. The most frequent characteristic of hibernation is lowering of body temperature as a consequence of low environmental temperature, acompained by inactivity, stagnation in growth etc. (Dugbartey et al. 2013). Hibernation is a complex phenological phenomenon during which torpor-physiological activity rhythm is synchronized with the seasonal rhythm and inner signals (Papović et al. 1985). Adjustments of the organism are manifested in both tissues and the system of organs as well as in the genes, proteins, protein complexes of the cell. Within the most important environmental stimulants for initiating hibernation and torpor, except food supply and environmental temperature is the day length which is considered a crucial outdoor signal for ectotherms, specifically snails. Photoperiod is the main cue that triggers supercooling ability in the land snail, *Helix aspersa* (Gastropoda: Helicidae) (Ansart et al. 2002).

During summer months some animals enter in the state of torpor which is called estivation. Gastropods are particularly exposed to estivation, because of the high sensibility to high temperatures (Holtz and Von Brand 1940). This process is very often present in snails because 78-92% of their body substance without the shell is consisted of water, so estivation is a favorable way to survive in conditions of low humidity (Elmslie 1998). To avoid evaporation of their body water (*i.e.* dehydration), they are active during high humidity, otherwise snails retract into the shell or synthesize calcareous epiphragm. In experimental conditions land snails can stay in estivation for many years which means that the decrease of the metabolic rate is not only connected to environmental factors like water availability or temperature regime but also depends on endogenous factors (endogenous circannual clock, antioxidant defence) (Nowakowska 2011). Because of constant possibility of dehydration snails store water or they keep free water in the mantel cavity which is used for moistening feet during the movement (Nowakowska 2011). Change of the amount of water depends on the concentration of Na^{+} and amount of the fraction of proteins as the carriers

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of osmotic pressure (Ebanks and Grosell 2008). The increase of osmotic pressure in body liquids occurs in cases of dehydration of the snail and in the presence of water enables fast rehydration (Elmslie 1998).

Helix pomatia is a widely spread species so it is exposed to a wide range of ecological factors which affect its distribution. The levels of metabolites in hemolymph highly reflect the physiological condition and the way of living during the year (Nicolai et al. 2011). A significant problem during the cold period of the year for the snail is the maintenance of water levels and ionic balance in the body (Zachariassen and Kristiansen 2000). Freezing is lethal for many organisms because ice formation physically destroys subcellular architectures and results in considerable outflow of water from the cells (Nowakowska et al. 2006). This is avoided by changes in the level and distribution of substances for ice forming, increasement in concentration of low molecular melted substances like polyhydroxyl alcohols (glycerol), sugar and the production of antifreeze proteins (Ansart et al. 2002). Glycerol and sugars are synthesized from large reserves of glycogen which are saved during the summer and autumn through the diet (Ansart et al. 2002).

The aim of research was to analyze biochemical status in the hemolymph of *H. pomatia* in order to understand biochemical and physiological adjustments and surviving during the hibernation. Also, we wanted to determine whether the change in concentrations of biochemical parameters is associated with decreasement in metabolic rate during hibernation and if that significantly contributes to the metabolic depression.

Materials and Methods

Collecting of specimens and experimental design

For this research we analyzed biochemical parameters in hemolymph of 30 individuals of Roman snail (*Helix pomatia*). Analyzed individuals were collected in Batunac area near Bratunac in Bosnia and Herzegovina (coordinates: 44^o 18’, 19^o 34’). The collecting area was about 1000 m^2. Snails were collected during period of hibernation and in period out of hibernation, therefore snails were categorized into following groups: beginning of hibernation (n = 10, group I), end of hibernation (n = 10, group II) and post-hibernation period (n = 10, group III, control group).

At the time of collection, Group I and Group II of snails were in the hibernation period, as it was indicated by the presence of the operculum and that they were buried in the ground. The timing of their sampling in terms of temperature regime and the length of day and night was also adequate for initiation of hibernation. The biochemical analysis of Group I snail hemolymph was conducted in November 2016 and for Group II in March 2017, immediately after awakening from hibernation. During period from November 2016 to March 2017, individuals from Group II were kept and maintained in simulated circumstances similar to outdoor environmental microclimate (in the cage buried in the ground and exposed to certain environmental factors). Group III of snails were collected during May 2017 *in situ*. This group of snails was kept in cages (5 snails in each cage) and reared under controlled conditions at temperature (22 ^{o}C) and relative humidity (around 80%) (Group III).

Extraction of hemolymph

For the extraction of the hemolymph the pericardial cavity was used. Before the process, the place of extraction is desinfected by 70% ethanol. Monoscope magnifier (Voyager 10-25 x 42) was used to remove the shell of approximately 10 mm^2 and after that 5 mm deep stab was done by needle (0.90 x 38 mm, 20G x 1 ½’’) through the pericardial cavity and 0.5 ml of hemolymph was collected. For biochemical analysis, hemolypmh was used immediately after collection.

Biochemical analysis

We analyzed biochemical parameters: total proteins, albumins, globulins and minerals (Ca^{2+} and K^+) by spectrophotometer (Spectronic 20 Genesys, model 4001/4; Thermo Scientific, USA). Total proteins were analyzed using the Biuret method (2095 C; SGM Italy) (Lubran 1978), while albumins were analyzed by bromocresol green method (BCG; Quimica Clinica Aplicada S.A.) (Brackeen et al. 1989). Globulins are obtained by subtracting the total proteins and albumins ($c_{(globulin)} = c_{(proteins)} - c_{(albumins)}$).

The concentration of calcium was obtained by CPC method (o-cresolphtalein complexone with R2 starter, SGM Italy) (Parentoni et al. 2001), while the concentration of potassium is analyzed by the turbidimetric method (SGM Italy) (Karaman et al. 1959).

Statistical analysis

Results of this research are presented as the mean value, standard deviation and range and were analysed using IBM SPSS 17.0. For intergroup differences analysis of variation (ANOVA) was used, while inner group differences were obtained by post hoc analysis. Pearson test was used for analysing the correlation between total protein concentration and concentration of albumins and globulins.

Table 1. Concentrations of biochemical parameters, ANOVA analysis and Post hoc test.

	Mean + stdv			Range			ANOVA
	I	II	III	I	II	III	Sig.
Total proteins (g/L)	16.90 ± 2.86 [b]	8.97 ± 2.44 [b]	45.94 ± 4.40 [b]	11.94-20.81	5.33-12.09	40.47-54.33	0.00*
Albumins (g/L)	3.21 ± 1.32 [b]	2.59 ± 0.50 [b]	1.28 ± 0.23 [b]	1.49-4.50	1.98-3.41	0.83-1.49	0.00*
Globulins (g/L)	14.79 ± 4.76 [a]	6.38 ± 2.06[a]	44.67 ± 4.40[b]	7.43-26.37	3.35-9.13	39.12-52.87	0.00*
Calcium (mmol/L)	4.27 ± 0.21 [b]	1.82 ± 0.19 [b]	31.22 ± 1.30 [b]	3.90-4.65	1.54-2.10	29.85-33.25	0.00*
Potassium (mmol/L)	2.70 ± 0.70 [b]	9.75 ± 0.33 [b]	4.75 ± 0.30 [b]	1.74-3.72	9.21-10.20	4.32-5.23	0.00*

*p <0.05 (ANOVA)
*a and b – post hoc test (b: the significant value among all test groups, p <0.01; a: value is not statistically significant, p >0,05)
I – beginning of hibernation; II – end of hibernation; III – post-hibernation period

Results

In general, concentrations of the total proteins, globulins and calcium increase during hibernation in comparison to post-hibernation period (Table 1).

The concentrations of potassium were higher at the end of hibernation. In the pre-hibernation period, the highest values of the total proteins, globulins and calcium were noted, as well as the lowest value of albumins. Significant differences were identified between all three groups. The post hoc analyses show significant differences between group II and III, except in case of globulin concentration.

During hibernation period, the concentrations of total proteins, albumin, globulin and calcium were reduced, while the concentration of potassium was very high at the end of hibernation in comparison to its beginning. In the post-hibernation period, extremely high values of total proteins, globulin and calcium and low values of albumin were detected. Values of potassium concentrations were very different throughout all periods and the highest values were estimated during the awaking period, *i.e.* at the end of hibernation. The post hoc test showed significant differences between three groups regarding analysed biochemical parameters, except for globulin values during hibernation.

There was a positive correlation between total proteins and albumins/globulins (Table 2).

Significant differences were estimated for total proteins and globulin concentrations between all groups, and significant differences regarding albumin concentrations were found when we compared Group I and Group II (beginning and end of hibernation).

Discussion

Suspended animation known as torpor is an outstanding evolutionary adaptation in animal kingdom, especially in gastropods. For example, snails (Gastropoda) close their shell aperture with a lid and inside the shell they withdraw, the resulting air cushions isolating them further against the cold. Significant changes in biochemical profile of their body fluids are also present during dormancy period. Our study showed changes in content of hemolymph during different period of time due to the exogenic factors (ambient temperature). Great changes of hemolymph content were notable in protein concentration. Accordingly, protein synthesis has been shown to be considerably reduced in types of metabolic depression that involve a physiological stress. Protein synthesis cannot be measured, but reactivation of translation on rehydration has been observed by (Wahba and Woodley 1984). Decreased concentration of total proteins, albumins and globulins were present during process of hibernation. High protein and globulin concentrations were obtained during post-hibernation period, while albumins had low values during same period. Decreased ingestion of food leads to lower amino acid amount in organism for protein synthesis. During hibernation, respiration in snails is suppresed, so hemocyanine as their respiratory pigment is present in low concentrations. According to Markl (2013), Lieb and Todt (2008) molecules of hemocyanin

Table 2. Pearson correlation between total proteins and albumins/globulins concentrations.

Season	Parameters	R	Sig.
Beginning of hibernation	Albumin	0,463	0,178
	Globulins	0,724	0.018*
End of hibernation	Albumin	0,803	0.005**
	Globulins	0,99	0.000**
Post-hibernation period	Albumin	0,297	0,404
	Globulins	0,999	0.000**

** Correlation is significant at the 0.01 level. Significant differences at the level p <0.01 (99% confidence interval and 1% absolute precision).
* Correlation is significant at the 0.05 level. Significant differences at the level p <0.01 (99% confidence interval and 5% absolute precision).
P values lower than 0.05 (p<0.05) were considered as significant and p-values lower than 0.01 (p<0.01) as highly significant.

are oligomers consisted of protein conglomerations which are made of functional subunits. Hemocyanine subunits are among the biggest polypeptides in nature. Hemocyanin in phylum Arthropoda is an agregate of molecules, while in molluscans hemocyanine consists of 7 to 8 different subunits connected with linker proteins (von Holde and Miller 1995). Accordingly, low protein concentration could be related with decreasement of hemocyanine concentrations during hibernation period due to low respiratory rate. Furthermore, the cupper metallothioneins (family of cysteine-rich, low molecular weight proteins) play an important role in metal homeostasis and hemocyanin synthesis in a cell type called rhogocyte in snails (Dallinger et al. 1997).

Since the shell has to be preserved during hibernation, the protein transport occurrs in the shell to maintain integrity, whereby the amount of protein in the plasma is reduced. Additionally, the decrease of water during hibernation raises the ratio of dissolved particles per volume of water so proteins and hemocyanin are decomposed (Stephenson and Lewis 2011). Since total proteins comprise albumin and globulin, the reduction of total protein concentration correlates with the reduction in albumin and globulin concentrations. Hibernation factors such as RMF, HPF, and YfiA turn off protein synthesis during hibernation process as reported by (Polikanov et al. 2012). The rate of protein synthesis reported by (Pakay et al. 2002), accounts for a significant proportion of the metabolic rate of the tissues in which it is measured. Relatively-low albumin values are the result of reduced protein synthesis during hibernation, while elevated globulins could be related with an increased need of immunoglobulins that are part of globulin protein family. High percentage of pathogens in snails food in post-hibernation period is present and this can be associated with a high concentration of immunoglobulins that play a role in the immune system. Large percentage of protein in hemolymph during post-hibernation period is the result of intense metabolic processes after hibernation. Concentrations of globulines, especially metalloprotein containing copper (hemocyanin) which weights vary from 300 to 9 000 kDa are increased due to high respiratiory rates (Lieb and Todt 2008). High protein concentration in snail meat and hemolymph are reported by Adegoke et al. (2010), Imevbore (1988), Saldanha et al. (2001). Babaloa and Akinsoyinu (2011) found high values of total proteins, low albumin values and low globulin values in *Archachati namarginata*. According to literature data (Babalola and Akinsoyinu 2011; Suljević and Mehinović 2014; Pedrini-Martha et al. 2016) albumin values are more dependent on the type of diet and rate of humidity compared to the globulin concentration. Albumins have many physiological roles in hemolymph as they participate as osmotic pressure carriers (Nowakowska 2011). Albumin also has antioxidant properties. Albumins regulate osmotic pressure, hence the degree of hydration. In this case, the shell has a protective role against dehydration, which causes a low value of albumin. On the other hand, calcium levels vary considerably based on type of diet. Calcium concentrations were increased and they were very high during the pre-hibernation period. Similar results were obtained in research Adegoke et al. (2010). Calcium is the most important element for the gastropod biology in general, including predator protection by creating a solid shell, adult survival, ovulation speed, egg and embryo development (Appleton 1978; Dawies and Erasmus 1984). It is involved in many enzymatic reactions and is required in metabolic processes associated with acid-base equilibrium (Sminia et al. 1977). Many physiological changes may affect the calcium reservoir in the shell and in the hemolymphs of molluscs (Souza et al. 2000). Due to the important role in biochemical processes, such high calcium concentrations are understandable. Low calcium concentration right after hibernation may be the result of additional need for reorganization of the shell and for metabolic needs. After period of hibernation, calcium concentration rapidly grows, as it plays many important roles in enzymatic activity, shell biomineralisation and repairing of cells (Lüss et al. 1998; Ikauniece and Jemeljanovs 2013). Soido et al. (2009) found even over 900 mg of $CaCO_3$/g in shell ash.

In the hibernation period the Ca^{2+} concentration is considerably lower since the epiphragm building requires the investment of these ions (Nowakowska 2011). The presence of calcium ions is also important for phagocytic hemocyte activity (Wilbur 1984), especially due to immunologic role of globulin (Zhang et al. 2004). Potassium has important role in osmoregulation and as a cryoprotectant. Potassium concentration was increased at the beggining of hibernation as a result of its cryoprotective function during winter and decreased during hibernation period. Lower potassium concentration beyond hibernation period were obtained in an invasive aquatic snail *Pomacea canaliculata* as repored by (Cueto et al. 2011). The changes in potassium concentration may be the result of different cell membrane permeability for Na^+ and K^+ ions during and after hibernating period which is already known (Stöver 1973; Meincke, 1975). Beyond hibernation period, potassium level rises in hemolymph due to its multiple role, especially in muscle contraction and heart rate regulation.

These are the first results regarding concentration of biochemical parameters in *H. pomatia* in context of hibernation as hypometabolic defense. Protein synthesis is the energy-utilizing process during metabolic depression and in our study decreasement of protein concentration in general has been shown during hibernation period. Total protein, globulin and calcium levels were decreased, while the concentration of potassium was evidently increased after hibernation.

Biochemical responses during hibernation period in snail are consequences of adaptations to low environmental temperature which allow tolerance and avoidance of death.

Study of seasonal biochemical patterns in relation to aspects of behavioural ecology in natural snail populations should be useful for understanding functional aspects of biochemistry and behaviour. These results serve us for better understanding of hibernation processes in exotherm animals. Also, studies on the physiological responses of *H. pomatia* to varying environmental conditions should provide information for establishing a biochemical parameters base before, during and after hibernation and for further research which concerns for conservation of this species.

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