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Hibernation perturbs the number of hemocytes and causes hematological turnover: basal traits to understand season-dependent physiological variations in *Helix pomatia* (Gastropoda: Helicidae)

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Abstract: The total number of hemocytes and their morphological characteristics were analyzed in *Helix pomatia* Linnaeus, 1758, by light microscopy during different phases of hibernation. Three types of hemocytes were identified: hyalinocytes, agranulocytes, and granulocytes. Total number of hemocytes decreased significantly during hibernation. Most intensive changes in cell size, morphology, and appearance of special structures in granulocytes happened during the hibernation period itself. Termination of hibernation coincides with very important granulocyte transformation and the appearance of specific gigantic short-lived cells. All of the changes in cell number and morphology are characteristics of different hibernation phases and have a major role in adaptive hypothermia. Granulocytes are hemocytes of great importance in immune response while specific functions of hyalinocytes and agranulocytes remain largely unexplored.

Key words: *Helix pomatia*, hemocytes, hibernation, hyalinocytes

A great success in the evolution of animal behavior and physiology is the ability to survive seasonally unfavorable conditions, especially when it comes to variable and extremely low temperatures (Roots, 2006). Environmental variations lead animals to a state of hibernation, which causes depression of metabolic and physiological processes, as well as a slow development and reproduction so that the animal can survive (Dugbartey and Henning, 2013). Life activities of the gastropods depend on environmental factors throughout the whole year. Shells have great importance because they reduce water loss during summer days. The formation of the calcareous epiphragm, with which they bind to substrates, is an additional protection mechanism of evaporation (Elmslie, 1998). Snail hibernation includes shell closing by the secretion of the epiphragm, while energy is gained from stored food reserves during this process (Holtz and Von Brand, 1940). In experimental conditions, aestivation can last for years, which suggests that the metabolic rate depends on both exogenous and endogenous factors (Nowakowska, 2011). Respiration is reduced during hibernation and about 10% of the fluid is lost, which affects heart rate, oxygen consumption, and energy requirements (Nicolai et al., 2011).

Metabolic changes during hibernation affect the value of circulating hemocytes. The phagocytic activity of hemocytes and lectins, as a defense mechanism, takes place thanks to the presence of calcium ions in the snail hemolymph. Consequently, the changes in the values of calcium ions affect the hemocyte number (Saleddin and Wilbur, 1984). Many studies confirmed the importance of hemocytes in immune responses, antibody recognition, phagocytosis, encapsulation, and cytotoxic reactions (Yoshino et al., 2001). Two types of hemocytes, granulocytes and hyalinocytes, have been identified in the hemolymph of *Biomphalaria* (Ratcliffe, 1985).

Seasonal factors and different experimental tests affect the total number, type, and behavior of hemocytes (Oliver and Fisher, 1995). The role of hemocytes today is most widely considered as an immunological response to infections. Seta et al. (1996) reported that the phagocytic potential of hyalinocytes is questionable and that only granulocytes are crucial in the immune response. Vinaud et al. (2008), Delgado et al. (2001), and Matricon-Gondran and Letocart (1999) identified four types of hemocytes based on their size (small, medium, large, and gigantic cells) in the hemolymph of *Biomphalaria glabrata*.

According to morphological criteria, there are two types, round cells and spread cells (Sminia, 1981), while in the study of Adamowicz and Bolaczek (2003), they refer to these cells as type I and type II. Wojtaszek et al.

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(1998) listed four types of hemocytes in the hemolymph of *Helix pomatia* based on morphological characteristics. Recently, using electron microscopy, Cueto et al. (2015) introduced a new classification of hemocytes into three types: hyalinocytes, agranulocytes, and granulocytes. Considering a large number of studies about hemocytes and their defensive role, we conducted this research during hibernation, when the immune response is less active and has a weaker response on pathogens, with the resulting different data for the total number, type, behavior, and production of hemocytes.

Since recent studies are limited to snails surviving during hibernation, there is little information about the types and roles of hemocytes in *Helix pomatia*, and the aim of this study is the identification and monitoring of hemocyte production during different periods of activity, including hibernation.

For study purposes, a total of 60 garden snails (*Helix pomatia* Linnaeus, 1758) were collected around Sarajevo at different times of the year. The area of snail collection included approximately 2000 m^2 with sea level altitude varying from 518 m in urban areas to 1164 m in hilly areas, respectively. Collected snails were all adults with foot and shell size variation (foot size mean = 10.4 cm; shell size mean = 4.12 cm). Three groups of snails were created: start of hibernation (n = 15, Group I), end of hibernation (n = 15, Group II), and posthibernation (n = 30, Group III). Hibernating specimens (n = 30; November 2017) were transported to the Biochemistry and Physiology Department of the Faculty of Natural Sciences and Mathematics, University of Sarajevo, Bosnia and Herzegovina (B&H). Living conditions (from temperature, moisture, and aeration to light/darkness cycles) were provided to imitate natural hibernation conditions for this species. The first group was immediately analyzed, while the second group was analyzed at the end of the hibernation cycle (March 2017). The snails from the third group (posthibernation) were collected from the same sites as previous groups and were quickly analyzed (May 2017).

To avoid mixed results and background particles, all specimens must be cleaned of secretions and residual hemolymph. Specimen shells were wiped with moist cotton (70% alcohol and distilled water) and dried with an absorbent tissue. Under medium magnification (15×; monoscope magnifier), incisions were made on shell surfaces (15 mm^2) using a small surgical scalpel (4 mm blade length; Semikem, B&H), after which a syringe (small-gauge needle, 0.9 mm; Semikem, B&H) was used for hemolymph extraction from the pericardial region (3–4 mm deep, just enough to penetrate the mantle). Special attention was paid to the angle of the needle and the depth to avoid hurting the animals and to cause less stress.

Approximately 50 µL of hemolymph per snail was collected and placed in ice-cooled containers prepared for the staining procedure. Collected hemolymph samples were taken from the ice, smeared across the microscopic slide, and left to dry (20 min). Afterward, they were stained with May–Grünwald–Giemsa stain (MGG; Semikem, B&H).

Hemolymph smear analysis was conducted on a light microscope (Olympus BX41) and photographs were taken by a mounted digital camera on the aforementioned microscope (Olympus DP-12). For cell counting, a Neubauer hemocytometer was used on a light microscope (Olympus CH20). Images of identified cell types were taken and processed via licensed software (Olympus DP-12 Soft; DP12-CB Ver.01.01.01.42, Olympus Corp.).

Main results from this study were represented as mean values with standard deviation (SD) and variance. Range values were added to differential hemolymph smear analysis. The variance was analyzed based on ANOVA and is represented by each data block. The post hoc Tukey test was used for analysis of intragroup differences.

Figure 1 represents the hemocytes identified during the hibernation period. During hibernation, three types of hemocytes are detected: hyalinocytes (Figure 1a), granulocytes (Figure 1b), and agranulocytes (Figure 1c). At the end of hibernation a new type of "transformed" cell is revealed (Figure 1d), probably caused by granulocytes. Hyalinocytes do not possess cytoplasmic granules; they have an eccentric nucleus that is usually elongated in shape. Granulocytes possess small eosinophilic granules. The highest number of them have a large, round central nucleus. Different stages of differentiation sometimes lead to similarities with agranulocytes, although they are much larger. Agranulocytes (Figure 1c) do not have cytoplasmic granules. The nucleus is often round. A particular type of cell occurs at the end of hibernation (Figure 1d). These are the most dominant and the biggest cells. The whole cell is usually like a thread, while in some other forms the central part can be seen as a bridge. This type of cell can be noticed only at the end of hibernation; they have a short life and on hemolymph smears the complete destruction of the cells occurs in several days, so they cannot be noticed. However, during hibernation, different forms of the nucleus appear (Figure 2), such that the cell changes its shape and size.

Figure 2 represents the most common hemocyte differentiation types (granulocytes) during hibernation. With light microscopy, normal forms of cells and a significant percentage of cells with increased size and amoeboid appearance were identified. First cells have a large, centrally set nucleus, with condensed cytoplasm. Mature forms are large cells with enlarged nucleus, which occurs with a foamy appearance.

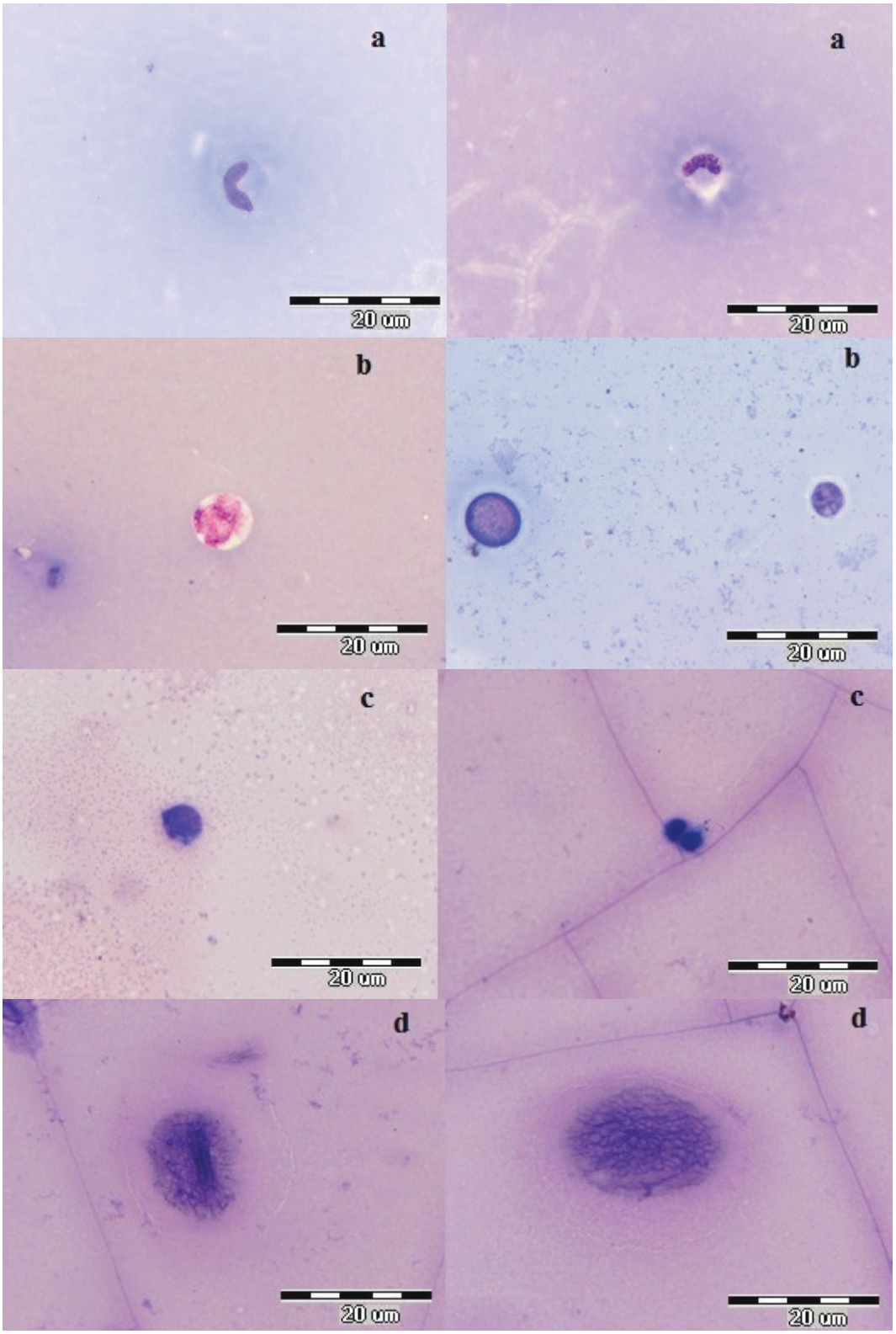

Figure 1. Circulating hemocyte types: a) hyalinocytes, b) granulocytes, c) agranulocytes, d) transformed cell, probably from agranulocytes (a–c, hibernation period; d, end of hibernation period).

Figure 3 shows the stages of transformation of hemocytes, probably granulocytes. As the initial cell, the granulocyte is an extremely large cell, and its characteristic is a reduced ratio of the size of the nucleus in relation to the cytoplasm. The transformation entails the destruction of the peripheral parts so that the nucleus in some stages can be seen as a bridge. The last picture shows the total transformation that occurs as filaments are scattered in the hemolymph.

In the Table, the percentage ratios of hemocytes including the mean, standard deviation, and range are presented. The ratio is presented for all three groups, and the last column indicates significant differences (ANOVA) between the groups.

During hibernation, the number of hyalinocytes is reduced, and in Group III it is increased. Agranulocytes have the highest percentage during hibernation, followed by granulocytes. In the posthibernation group (Group III), granulocytes are the most represented cells. The transformed cells are only represented at the end of hibernation. For all three types of hemocytes, a significant difference was established. The post hoc test showed significant intragroup differences for hyalinocytes and granulocytes. Intragroup differences for agranulocytes between Groups II and III were not determined.

Mollusk hemolymph analysis is still in its primal research stages. Hemolymph of snails, as a functional medium, is susceptible to physiological changes that are usually induced by biotic (species, age, sex, and physiological status) and abiotic factors (environmental processes). Three types of cells were observed in the garden snail's hemolymph: granulocytes, agranulocytes, and hyalinocytes. Many research sources exist today on hemocyte types in mollusks. A unique classification has not been achieved yet, because there are various obstacles in the way. Karuthapandi (2010) reported two types of hemocytes, granulocytes and agranulocytes, in *Achatina fulica*. It was concluded that granulocytes were the most numerous fraction of hemocytes, which we also confirmed in our research for the garden snail in Group III. Cheng and Auld (1977), as well as Cheng (1975), found that granulocytes were the predominant type of phagocytes. Based on cell size, shape, and nucleocytoplasmic ratio along with different staining techniques, three subtypes were

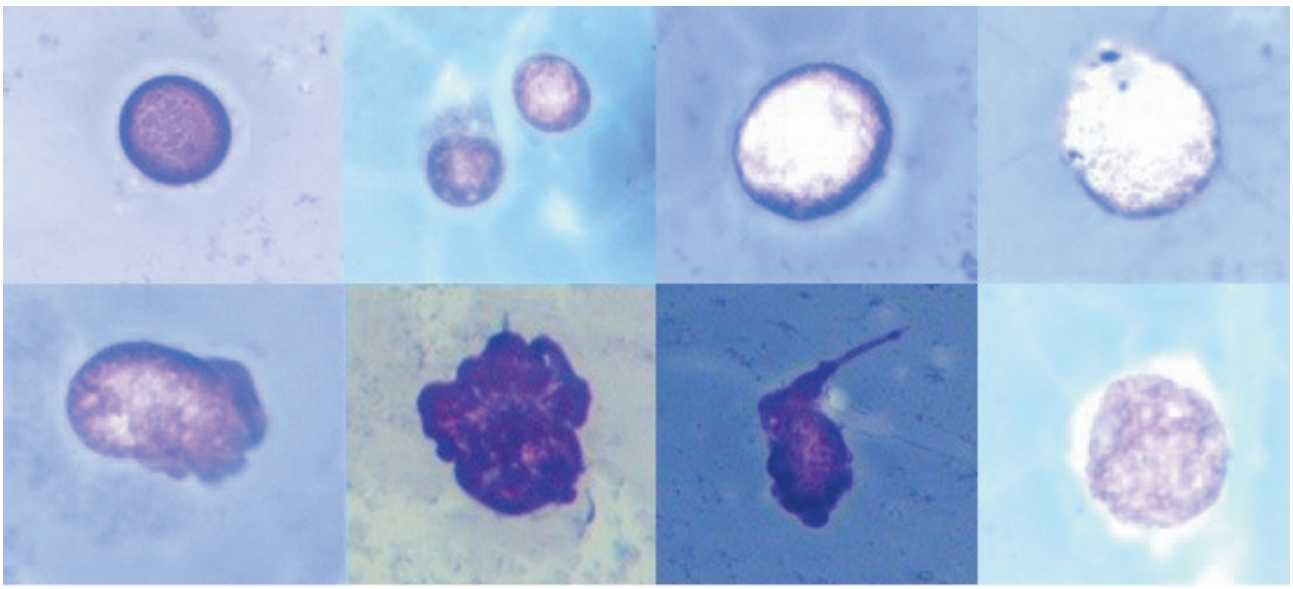

Figure 2. Different stages and granulocyte forms during hibernation.

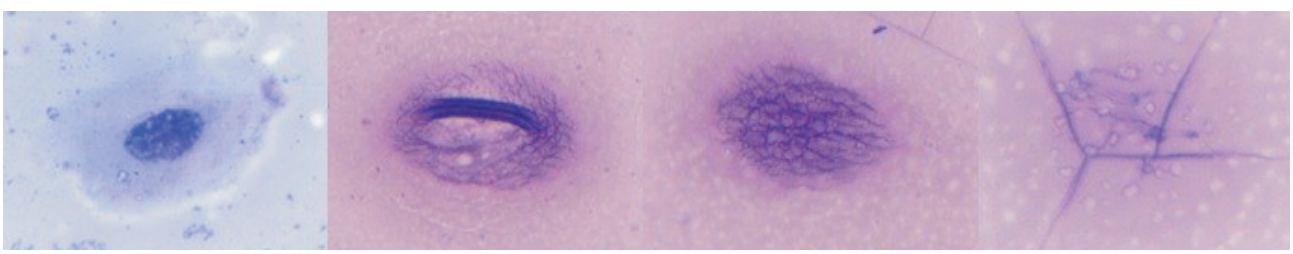

Figure 3. Transformation of hemocyte type (probably granulocytes) to a new type of cell.

Table. Percentage ratios of hemocytes, and ANOVA and Tukey post hoc test results.

Type of cell	Group I		Group II		Group III		ANOVA
	Mean ± SD	Range	Mean ± SD	Range	Mean ± SD	Range	Sig.
Hyalinocytes	10.80 ± 2.66[a]	7–15	4.70 ± 1.76[b]	2–7	21.5 ± 2.41[c]	18-26	0.00*
Agranulocytes	49 ± 8.51[a]	37–62	19.60 ± 3.33[b]	15–25	20.7 ± 2.83[b]	17-25	0.00*
Granulocytes	40.20 ± 8.20[a]	30–53	14.40 ± 2.41[b]	10–17	57.8 ± 4.15[c]	50-65	0.00*
Transformed cells	-	-	61.3 ± 4.90	55–68	-	-	-

* Significantly different ($P < 0.05$).
[a,b,c] Post hoc test. Values in the same row for the same parameter among different groups that do not have the same superscript letter showed statistical differences.

determined, I, II, and III, from *Trachea vittata* (Mahilini and Rajendran, 2008) and *Achatina fulica* (Karuthapandi, 2010). Changes in numbers, morphology, and types of hemocytes could appear as a result of the interaction of abiotic and biotic factors (Suresh and Mohandas, 1990; Suljević et al., 2018). McCormick-Ray and Howard (1991) correlated changing hemocyte types to physiological needs of the organism *Crassostrea virginica*.

Abiotic factors, along with the physiological needs of an organism, do have a very potent effect on changes in numbers and morphology of hemocytes, which we observed in this research. Although changes in hemocyte morphology are noticeable in all types, the most significant ones are in granulocytes, as they are the most numerous hemocyte type. Hibernation leads to a gradual decrease in the number of granulocytes, which at the end of hibernation remains quite low.

Additionally, granulocytes go through the most evident morphological changes, varying from cell size changes to pseudopodia formation, which could lead to cell misidentification. At the end of hibernation, a new special gigantic type of cells appears, which we assume are transformed granulocytes. Their life expectancy is quite short and they degrade rapidly. We have established that the granulocyte number gradually decreases during hibernation. The appearance of gigantic cells coincides with the period of awakening from hibernation, which can be a mechanism for the cleaning of old cells. A low number of granulocytes during hibernation is probably a consequence of reduced immune response. This slowed immunologically response can be the result of nutrient deprivation and fewer ingested antigens as it is a period of reduced activity.

Granulocytes have an important function in the general immune response. Soderhall (1982) considers granulocytes to contain prophenoloxidase in their granules. This enzyme is needed for the melanization process as well as foreign body recognition. In in vitro conditions, granulocytes perform phagocytosis on bacteria and mold hyphae. Smaller granulocytes perform phagocytosis while larger granulocytes perform encapsulation (Hose and Gary, 1989). Mitosis was observed in hyalinocytes and smaller granulocytes, which suggested the existence of two cellular lineages (Martin et al., 1987). Hemocyte aggregation in damaged or infected tissue causes improved tissue healing and foreign body phagocytosis (Ratcliffe and Rowley, 1979). Encapsulation represents a type of cellular defense mechanism in which the invading organism is surrounded by small and large granulocytes with formation of fibrin. Coagulation protein (CP) or coagulogen is critical for proper coagulation (Martin et al., 1991). Polymerization of CP protein starts with transglutaminase activation, which occurs within hemocytes themselves (Florkin, 1960), creating protein aggregations. The coagulation process is slowed down by low temperature (Dean and Vernberg, 1966), so the low number of granulocytes is confirmed during hibernation.

A sudden increase in granulocyte number is noted after the moment of awakening from hibernation. This rapid granulocyte production mechanism remains unclear. However, residues of degraded gigantic cells are observed in hemolymph and could be linked to initial factors for rapid granulocyte production. The number of hyalinocytes is in general very small, especially during the hibernation period. The physiological role of hyalinocytes is not entirely understood. Easily degraded, this type of cell is very sensitive to tension forces (especially, for example, a foreign body) and could easily be disintegrated, releasing cytoplasm and destroying the cell membrane. Hyalinocytes are considered to have an active role in coagulum formation (Hardy, 1982). Vacca and Fingerman (1983) proposed that hyalinocytes create diphenolic proteins for shell colorings while granulocytes contain proteins. It has been observed in some crustacean species that hyalinocyte number increases after molting (5–10 h) and stabilizes after 1–2 days, while granulocyte number increases before molting.

Increased hyalinocyte number could be connected to shell renewal and its coloration, which is considered a very important phenotypic adaptation to living conditions. The number of agranulocytes is similar to the number of hyalinocytes. Also, decreased number of agranulocytes is evident during hibernation but is not as low as the number of hyalinocytes.

No significant morphological changes were observed in agranulocytes during hibernation. The roles of agranulocytes are still very vague. In vitro phagocytosis has revealed the interesting fact that all hemocytes possess a certain level of phagocytosis. If exposed to fluorescent beads, certain hemocyte populations like hyalinocytes and agranulocytes actually retain most of their morphological characteristics while granulocytes become modified. This modification includes degranulation and vacuolization of cytoplasm, condensed nuclei, and creation of short filopodia (Cueto, 2015).

In conclusion, analysis of hemocytes in hemolymph, as an extravascular fluid with several crucial functions in garden snail *Helix pomatia* and Mollusca in general, is very important in understanding their physiology. The obtained results on the types and numbers of hemocytes in different seasonal phases help in the assessment of the immune system in animals and also improve their success in adaptation to the environment. Differences in hemocyte production and degradation patterns coincide with different hibernation phases, which could represent an adequate model for adaptive hypothermia research in other organisms.

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