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Determination of selected biochemical parameters of the haemolymph of free-living *Cepaea nemoralis* (L.) snails. Preliminary study

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Summary

The study objective was to determine reference values of selected biochemical parameters of the haemolymph of *Cepaea nemoralis* snails (*Mollusca*, *Gastropoda*, *Helicidae*). The study determined the activity of aminotransferases (AST and ALT), which was 11.22 ± 10.92 [u/l] and 6.72 ± 3.18 [u/l], respectively, and alkaline phosphatase (ALP) at a level of 3.92 ± 2.08 [u/l]. The level of amylase (AMY) in the haemolymph was 2.32 ± 0.74 [u/l], while lipase (LIP) was 0.74 ± 0.79 [u/l]. The concentration of urea (UREA) was established at 2.28 ± 0.48 [mg/dl], creatinine (CREA) at 2.28 ± 0.09 [mg/dl], uric acid (UA) at 0.22 ± 0.16 [mg/dl], calcium (Ca) at 21.24 ± 2.4 [mg/dl], and total protein (TP) at 1.38 ± 0.34 [g/l]. The specific gravity of the haemolymph was 1.0 ± 0.002. The haemolymph was obtained from 211 individuals in the active phase collected from their natural habitat. The biochemical parameters of the haemolymph were determined with a BS 180 apparatus (Mindray), and the specific gravity with a refractometer. The article also desrcibes an intravital technique of collecting haemolymph from *C. nemoralis*. The results obtained may constitute an aid and a guideline for scientists dealing with the physiology of land molluscs.

Keywords: *Cepaea nemoralis*, haemolymph, biochemical parameters

The grove snail (*Cepaea nemoralis*) is a pulmonate land snail of the Stylommatophora order, family *Helicidae*, genus *Cepaea*. Natural habitats of *C. nemoralis* are found throughout the Western and Central Europe. The northern boundary of the *C. nemoralis* distribution is located in Scotland and southern Scandinavia, and the southern boundary in the Mediterranean region. The natural range of *C. nemoralis* includes the western part of the Iberian Peninsula, as well as Ireland and Great Britain (4). The species has also been able to colonise new areas, including coastal regions of the Baltic Sea, where it is considered an introduced species (17). Currently, there are numerous areas in Central and Eastern Europe where *C. nemoralis* has been introduced by humans (9, 11). *C. nemoralis* was also introduced into North America in the 19th century, where it is present, among others, in the north-eastern states of the United States (3). The snail is active from spring through autumn, particularly during rainy weather. On hot days and under drought conditions, it may enter the estivation state, whereas from late autumn to early spring it hibernates under a layer of plant and soil material (19). *C. nemoralis* is found in a variety of habitats, including meadows, gardens, parks, cemeteries, forests and peat bogs. Its diet consists mainly of dead and live plant material, carrion, fungi, peat and algae (20, 21). Its shell has a width of 18-25 mm (rarely 30 mm), while its height ranges between 12 and 22 mm (rarely above 25 mm). The shell can be of brown, orange, red, yellow or olive colour and feature from one to five black or dark brown spiral stripes, which may fuse or be absent. The shell colour variability in *C. nemoralis* is genetically determined. The lip of the shell is clearly marked, violet-brown, thickened and slightly curved. The body of the snail is of creamy colour, the foot and the head are darker (6, 7, 10).

C. nemoralis is a hermaphrodite with cross fertilisation, which takes place several times a year. After fertilisation, the snail lays eggs in the form of a cocoon into a pit dug in the ground. The egg package contains from 30 to 80 eggs, and their diameter ranges between 2.3 and 3.0 mm. The juveniles hatch within 15-20 days (22). The life expectancy of wild *C. nemoralis* reaches up to six years. *C. nemoralis* snails have been used in environmental studies, and the correlation between their physiological parameters and their variable environment has been a subject of research (13, 14, 16). In this context, it seems to be particularly important to determine the physiological parameters of the haemolymph of *C. nemoralis* collected directly in their natural habitat. The haemolymph of land snails, apart from the basic role of the body fluid responsible for respiratory and immunological processes, forms the so-called hydrostatic skeleton responsible for maintaining the appropriate cell turgor and for some movement elements related to the pumping of haemolymph to specific areas of the body. For this reason, the volume of haemolymph in a land snail's body is relatively high, which made it possible to obtain an appropriate amount of haemolymph for purposes of this study. Previous studies in which the biochemical parameters of land snail haemolymph (*Cornu aspersum aspersum*, *Achatina fulica*, *Cepaea nemoralis*) were determined used apparatuses for the biochemical assessment of mammalian blood serum (23-25). The use of analogous methods makes it possible to obtain repeatable values of the parameters analysed.

Fig. 1. Method of haemolymph collection from *Cepaea nemoralis* snail

Material and methods

The study material consisted of haemolymph drawn from 211 *C. nemoralis* snails. The snails were collected in the period from September to October 2019 in the vicinity of Lublin (south-eastern Poland) in meadow areas. Only active animals were collected, with the fact of feeding in a humid environment being regarded as a sign of the normal functioning of the organism. The snails were collected in the morning hours, between 5:00 and 6:00 a.m. The air temperature ranged between 9 and 14°C. After the snails were transported to the laboratory, they were placed in collective containers with ventilation openings, where they remained for 1 to 2 hours at a temperature of 20°C, after which they were washed and cleaned of outer shell impurities. After cleaning, haemolymph was collected. In the case of *C. nemoralis*, the technical difficulty in collecting haemolymph consists in obtaining the maximum amount of material from the relatively small-sized molluscs. Adaptation of the method for haemolymph collection from the main pulmonary vessel was technically very difficult due to the small body size of *C. nemoralis*. The procedure applied in this study consisted in inserting a needle with a diameter of 0.7 mm and a length of 40 mm, with the plastic needle hub removed, into the central-posterior dorsal part of the body when the body of the snail was protruding from the shell (Fig. 1). Out of 211 *C. nemoralis* snails from which haemolymph was collected by this procedure, only 3 individuals died. The efficiency of this method is relatively high, as haemolymph was successfully drawn from 92% of the snails, that is, 194 individuals.

The amount of haemolymph collected from one individual ranged between 0.1 and 0.25 µl. In the next stage, the haemolymph was centrifuged for 1 min at 3000 rpm to separate haemocytes. The supernatant was collected for further biochemical determinations. To perform the determinations, a BS-180 (Mindray) apparatus for blood biochemistry testing was used. This apparatus is routinely used in analytical laboratories in human and veterinary medicine to evaluate biochemical parameters in human and animal blood serum. The authors have also successfully utilised it to examine snail haemolymph in previous studies (23-25). In the haemolymph collected in the present study, the activity of aminotransferases (AST, ALT), alkaline phosphatase (AP), amylase (AMY) and lipase (LIP) was determined, and the concentration of urea (UREA), creatinine (CREA), uric acid (UA), calcium (Ca) and total protein (TP) was determined. The values obtained are presented in Table 1. Due to the small amount of haemolymph collected from one snail (mean 0.15 µl), only selected parameters, typically 2-4, were determined from each sample. The results are a list of values obtained from all determinations performed. The mean values of the parameters were determined on the basis of at least 20 replications, whereas haemolymph was successfully collected from a total of 194 snails. Moreover, the AST/ALT ratio for the haemolymph of the animals was determined. Likewise, the ratio between urea and uric acid concentrations was calculated. A portion of the haemolymph was also used to determine its specific gravity with a refractometer. The results obtained were subjected to basic statistical analysis, and the arithmetic mean and standard deviation were calculated.

Results and discussion

This research into terrestrial snails *C. nemoralis* was undertaken because of the increasing role of snail as a source of protein-rich food and as intermediate hosts

Tab. 1. Results of the examination of selected biochemical parameters and specific gravity of the haemolymph of *C. nemoralis* snails

Parameter	Number of samples tested	x̄ ± SD	Minimum	Maximum
Specific gravity	20	1.0 ± 0.002	1.006	1.014
AST [u/l]	23	11.22 ± 10.92	1.72	54.86
ALT [u/l]	21	6.72 ± 3.18	2	13.42
AST/ALT	21/21	1.54 : 1	0.46 : 1	4.8 : 1
UREA [mg/dl]	23	2.28 ± 0.48	2.15	2.36
CREA[]	24	0.28 ± 0.09	0.23	0.71
UA [mg/dl]	21	0.22 ± 0.16	0.11	0.72
UREA/UA	23/23	62.48 : 1	200 : 1	6 : 1
ALP [u/l]	21	3.92 ± 2.08	1	9.57
AMY [u/l]	21	2.32 ± 0.74	2	2.54
LIP [u/l]	22	0.74 ± 0.49	0.1	3.89
TP [g/l]	21	1.38 ± 0.34	0.13	1.91
Ca [mg/dl]	21	21.24 ± 2.4	17.83	25.86

of a variety of parasitic worms, as well as for academic reasons (12). In-depth knowledge of the physiological, ecological and eco-physiological aspects of terrestrial snails is not only crucial in protecting snails from various hazards in the environment, but also vital in human and animal health protection. The present study determined physiological norms for the selected biochemical parameters of the haemolymph of the free-living *C. nemoralis*. Most of the parameters determined are important in assessing the physiological status of the snail. It is well known that seasonal changes, the reproductive cycle and short-term changes in the environment, such as temperature and food availability, can change the concentration of haemolymph components (1). Since haemolymph is the internal medium with which all tissues of the organism communicate, even the slightest changes in tissues are reflected in the haemolymph as changes in its parameters. In the present study, care was taken to standardise the environmental factors, and the values obtained reflected the physiological parameters of active snails. In this study, the AST level was on average 11.22 ± 10.92 [u/l], and it was almost twice as high as the mean ALT level, which was 6.72 ± 3.18 [u/l]. The levels of these enzymes were characterised by very high variability. The available literature data indicate that the activity of AST and ALT and the levels of UREA and CREA in snails increase in the active phase, but decrease during hibernation, estivation and starvation (5). ALT and AST are key enzymes of amino acid metabolism that link amino acids to intermediates of pathways involved in energy generation. It is expected that increased energy demand during the active live cycle will result in the mobilisation of potential energy sources, including amino acids, resulting in the activation or induction of enzymes of amino acid metabolism (1). AST and ALT are also considered to be markers of muscle dystrophy and myocardial and internal organ damage. It is also known that these enzymes are present principally in cytoplasm and are secreted into blood after hepatocellular injury, which increases their levels in haemolymph (15).

The urea level established in the snails was 2.28 ± 0.48 [mg/dl]. The level of urea depends on amino acid metabolism and can increase as a result of increased activity of the snail. Urea is also known to be a good predicator of kidney disorder as the urea level in blood increases. In our study, the creatinine level was 0.28 ± 0.09 [mg/dl]. Creatinine is considered to be a waste product formed in the muscle from a high energy storage compound: creatinine phosphate. The muscular activity of an active snail can lead to the breakdown of this energy compound at a faster rate than the kidney can handle, which may explain the highest concentration of creatinine in the haemolymph. Uric acid in the snails was at a level of 0.22 ± 0.16 [mg/dl]. The uricotelism is characteristic of gastropods in general. It is known that uric acid in land snails is not related to the condition of their environment, but might depend on the frequency of excreting and general nitrogen metabolism.

Amylase and lipase are enzymes responsible for the breakdown of macromolecular compounds in snails' digestive tracts. The role of amylase is to break down complex sugars, such as starch and cellulose, whereas lipase has a lipolytic activity. Amylase is known to be present in snails' oral and intestinal mucus, but lipase has only been confirmed in their digestive tract (2, 8). Digestive enzymes are produced in the salivary glands and the hepatopancreas, also called the digestive gland. The digestive gland is composed of three cell types: digestive cells concerned with the absorption and digestion of nutrients, secretory cells that produce enzymes, and undifferentiated cells. Damage to and disorders of the hepatopancreas are associated with an increasing level of digestive enzymes in the haemolymph. The level of amylase established in this study was 2.32 ± 0.74 [u/l], and level of lipase was more variable, amounting to 0.74 ± 0.49 [u/l]. Alkaline phosphatase, in general, catalyses the hydrolysis of phosphomonoesters to produce inorganic phosphate. The specific function of ALP is unclear, but it is suggested that the biological role of ALP is related to trans-membrane transport processes (18). The haemolymph of *C. nemoralis* contained 3.93 ± 2.08 [u/l] of ALP, whose level was highly variable. The values of parameters presented in this study can be used as a point of reference for further research on the effects of external factors on the physiological parameters of *C. nemoralis* snails. The results prove that the intravital collection of haemolymph from *C. nemoralis* is possible. The method presented by the authors is easy to implement, efficient and relatively safe for the animals examined.

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