Polish Journal of Veterinary Sciences Vol. 21, No. 3 (2018), 445–449

DOI 10.24425/122617

Original article

Study on establishing normal ranges of chosen biochemical parameters of haemolymph of *Cornu aspersum maxima* and *Cepaea nemoralis* gastropods

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Abstract

The aim of the study was to establish normal ranges for chosen biochemical parameters of haemolymph of snails (Gasropoda: *Mollusca*), in the light of the use of these animals as experimental models in various types of studies. The study was conducted on 100 specimens of *Cornu aspersum maxima* (CAM) and 100 specimens of *Cepaea nemoralis* (CN). The haemolymph collected from the animals was analysed using colorimetry to assay aspartate transaminase (AST) activity, alanine transaminase (ALT) activity, amylase activity and the concentrations of urea and triglycerides. In the further part of the study, the influence of administering doxycycline with feed on the change of AST and ALT activity in snail haemolymph has been studied. The normal values established for CAM are as follow: AST activity: 26-38 u/l, ALT activity: 0-11 u/l, amylase activity 9-16 u/l, concentration of urea: 3-6 mg/dl, concentration of triglycerides: 16-20 mg/dl. For CN, the following data have been obtained: AST activity: 30-80 u/l, ALT activity: 0-15 u/l, amylase activity 12-15 u/l, concentration of urea: 5-8 mg/dl, concentration of triglycerides: 18-24 mg/dl.

It has been shown that doxycycline presents a high workload on the hepatopancreas of snails, which is reflected by a statistically significant ($p<0.05$) increase of AST and ALT activity in the haemolymph of the specimens which obtained doxycycline in feed, as compared to the groups with antibiotic-free feed. The haemolymph activity of both studied parameters increased together with study time and tetracycline administration time.

Key words: Cornu aspersum maxima, Cepaea nemoralis, biochemical analysis, haemolymph

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Introduction

Cornu aspersum (Helix aspersa) maxima (garden snail) is a snail species coming from the southern part of the Mediterranean Basin. It is characterised by fast body weight gain and its farming cycle lasts less than 12 months – a few times shorter than that of the Burgundy snail (*Helix pomatia*). The shell of *Cornu aspersum* is brownish grey and measures 2.5-4 cm in diameter (Ziomek et al. 2017).

The grove snail (*Cepaea nemoralis*) is a common synanthtropic species of snail living in moderate climates. It is characterised by a colourful shell, very often with dark bands. The verge of the shell also has a dark band. The diameter of the shell ranges from 1.7 to 2.7 cm (Cameron 2001). Both species of snails are highly resistant to environmental conditions, and therefore they are relatively easy to breed. Due to their edibility, the monitoring of their health condition constitutes an element of public health protection. Furthermore, ease of culturing and the current binding restrictions on research on vertebrates make them an attractive alternative model for various types of studies on living organisms. Therefore, it is necessary to determine and establish normal ranges of physiological parameters in snails, which will enable the monitoring of their health condition and be a reference in studies conducted on these invertebrates.

The aim of this paper was to establish normal ranges for chosen biochemical parameters of the haemolymph of the selected snails, in the light of their use as experimental models in diverse types of studies.

Materials and Methods

The study was conducted on 100 specimens of *C. aspersum maxima* (CAM) (weight: 28.5-38 g) and 100 specimens of *Cepaea nemoralis* (CN) (weight: 2.0-3.1 g) from our own experimental heliculture. The animals were fed with specialist feed for edible snails and kept in clean plastic enclosures with a size 20 cm × 30 cm × 20 cm (10 specimens of *C. aspersum maxima* per tank, and 25 specimens of *Cepaea nemoralis* per tank) in optimal temperature and humidity conditions (temperature: 20°C, humidity: 80-90%). The period of lighting was 10 hours. The haemolymph was collected from living snails using a method developed by the Department of Epizootiology and Clinic of Infectious Diseases, Faculty of Veterinary Medicine of the University of Life Sciences in Lublin (patent P.410296) (Ziętek et al. 2017). The volume of collected haemolymph was 0.3 - 0.6 ml from CAM and 0.18 - 0.2 ml from CN.

The collected haemolymph was centrifuged (1000 rpm, 1 minute) at room temperature and the obtained material was analysed using colorimetry (Mindray BS-130) for the following parameters: activity of aspartate transaminase (AST), alanine transaminase (ALT), and amylase (AMYL), as well as the concentration of urea (UREA), and triglycerides (TG). While establishing the normal ranges of biochemical parameters of haemolymph, extreme values were discarded. Extreme values constituted less than 10% of obtained results, were clearly different from other results, and were probably related to bad health condition of the given specimens (40% of them died within two months). Furthermore, the AST:ALT ratio was calculated for all animals studied.

In the further part of the study, the influence of administering doxycycline (200 mg/1 kg of feed) with feed on the change of AST and ALT activity in snail haemolymph was studied. CAM and CN were divided into 5 groups, 20 specimens each, designated as follows: *C. aspersum maxima* – groups H1, H2, H3, H4, and H5, *C. nemoralis* – groups C1, C2, C3, C4, and C5. The haemolymph from the snails belonging to groups H1 and C1 was collected on Day 0 of the study, before administration of feed with the antibiotic. The snails from other groups underwent haemolymph collection at 7-day intervals: animals from groups H2 and C2 - after 7 days from the beginning of the experiment; animals from groups H3 and C3 - after 14 days; animals from groups H4 and C4 - after 21 days, and animals from groups H5 and C5 - after 28 days.

Statistical analysis

The obtained results were analysed statistically by calculating the arithmetic mean and standard deviation, which then served for establishing the proposed normal ranges of the studied parameters. The lower limit of the normal range was calculated as arithmetic mean minus standard deviation and the upper limit as arithmetic mean plus standard deviation.

In the second part of the study, research into the influence of doxycycline on chosen parameters (AST and ALT) in both studied mollusc species, the analysis was performed using Student's t-test for independent samples where X1 was the average of results on Day 0 while X2 was the average of results on Day 28 (end of experiment). On the basis of the obtained results, the p-values were calculated.

Results

On the basis of the performed experiments, it was established that the following values may be assumed as

Table 1. Established norms of chosen biochemical parameters of haemolymph of *C. aspersum maxima.*

Parameter	AST u/l	ALT u/l	UREA mg/dl	TG mg/dl	AMYL u/l
Arithmetic mean of the obtained results	31.89	3.74	4.45	18.7	11.6
Value range after discarding extreme values	26-38	0-11	3-6	16-20	9-16
Percentage of results within the proposed normal range	96.5%	92%	98%	100%	100%

Table 2. Established norms of chosen biochemical parameters of haemolymph of *C. nemoralis.*

Parameter	AST u/l	ALT u/l	UREA mg/dl	TG mg/dl	AMYL u/l
Arithmetic mean of the obtained results	48.6	8.7	5.4	20.5	13.4
Value range after discarding extreme values	30-80	0-15	5-8	18-24	12-15
Percentage of results within the proposed normal range	96.9%	93.8%	99%	98%	100%

Table 3. AST:ALT ratio in haemolymph of studied specimens of *C. aspersum maxima.*

Parameter	Numeric values
AST:ALT ratio (average values)	8.5:1
Range of AST/ALT ratio in studied animals	14:1 - 5:1
Percentage of results within the proposed normal range	98%

Table 4. AST:ALT ratio in haemolymph of studied specimens of *C. nemoralis.*

Parameter	Numeric values
AST:ALT ratio (average values)	5.6:1
Range of AST/ALT ratio in studied animals	12:1 - 3:1
Percentage of results within the proposed normal range	98%

the normal values of the chosen biochemical parameters in CAM: AST activity: 26-38 u/l, ALT activity: 0-11 u/l, amylase activity 9-16 u/l, urea concentration: 3-6 mg/dl, triglyceride concentration: 16-20 mg/dl. For CN, the values are the following: AST activity: 30-80 u/l, ALT activity: 0-15 u/l, amylase activity: 12-15 u/l, urea concentration: 5-8 mg/dl, triglycerides concentration: 18-24 mg/dl. Depending on the studied parameter, the ranges specified in this way encompassed results obtained from 92% to 100% of the specimens of CAM and from 93.8% to 100% of the specimens of CN. The greatest range of results was observed during the establishment for the normal range for ALT (92% of the results for CAM and 93.8% of the results for CN fell within the proposed normal range, with a standard deviation of 7.26 and 6.3, respectively. The assayed ALT value was 0 in 11% of CAM and 9% of CN). As far as the AST activity in *C. aspersum* is concerned, the range of results was smaller (standard deviation: 6.11), while as much as 96.5% of obtained results fell within the proposed normal range. In CN, the standard deviation was very high (31.4). However, the normal range specified using the aforementioned method encompassed 96.9% of the results. The established normal ranges for the remaining parameters encompassed from 98% to 100% of the results, while the standard deviation of the results from the arithmetic mean did not exceed 4. The AST:ALT ratio in the described species was calculated in a similar way (arithmetic mean + the range of values considered as normal obtained after adding (upper limit) and subtracting (lower limit) the standard deviation. In the case of both snail species, the proposed normal range encompassed 98% of AST:ALT ratio results) (Tables 1 and 2). While the established normal ranges for ALT, amylase, urea and triglycerides were similar in both snail species, it is important to note quite large differences in the normal AST activity, as its physiological upper limit is twice as high in CN as in CAM. This is reflected in the AST:ALT ratio which amounted to 5.6 (average) for CN and 8.5 (average) for CAM (Tables 3 and 4).

In the second part of the study, it was shown that doxycycline is an antibiotic that presents a high workload on the hepatopancreas of snails, which is reflected by a statistically significant increase of AST and ALT activity in the haemolymph of the specimens which

Table 5. Results of biochemical study of serum of *C. aspersum maxima* which received feed with doxycycline.

Study group	Study day	AST (average values)	ALT (average values)	AST:ALT ratio (average values)	% of snails, the AST value of which fell within the range established by the Authors (Table 1)	% of snails, the ALT value of which fell within the range established by the Authors (Table 1)	% of snails, the AST:ALT value of which fell within the range established by the Authors (Table 3)
H1	0	32.95	4.6	6.4:1	96.9%	93.8%	98.5%
H2	7	36.5	5.8	5.2:1	96%	91.5%	98%
H3	14	42.3	8.2	6.1:1	92%	89%	95%
H4	21	53.9	10.3	8:1	86%	76%	86%
H5	28	58.2	11.4	11.5:1	74%	72%	82%

Table 6. Results of biochemical study of serum of *C. nemoralis* which received feed with doxycycline.

Study group	Study day	AST (average values)	ALT (average values)	AST:ALT ratio (average values)	% of snails, the AST value of which fell within the range established by the Authors (Table 2)	% of snails, the ALT value of which fell within the range established by the Authors (Table 2)	% of snails, the AST:ALT value of which fell within the range established by the Authors (Table 4)
C1	0	46.6	9.0	5.1:1	96%	93.4%	98.2%
C2	7	48.5	10.2	5.8:1	94%	91.5%	94%
C3	14	52.3	11.4	6.4:1	90%	88%	89%
C4	21	54.6	14.3	6.9:1	85%	78%	84%
C5	28	58.4	16.4	7.2:1	69%	68%	78%

received doxycycline in feed (groups H2-H5 and C2-C5), as compared to groups which do not receive the antibiotic (H1 and C1). The p-values for AST and ALT in CAM were $p=0.0416$ and $p=0.0389$, respectively, while for CN they were $p=0.048$ and $p=0.0465$, respectively.

Moreover, the longer the experiment time and tetracycline administration time were, the greater the activity of both studied parameters was (Tables 5 and 6).

Discussion

The results of the research enabled the determination of physiological normal ranges for two cultivated gastropod species: *C. aspersum maxima* and *C. nemoralis.* The importance of both species grows as they are cultured for consumption (Cameron 2001, Ziomek et al. 2017), used as bioindicators of environmental pollution (Bislimi et al. 2012) or used in research into the toxicity of various substances, including drugs (Ziętek et al. 2017). In earlier studies (Ziętek et al. 2017), there were attempts at establishing the physiological norms for chosen biochemical parameters of haemolymph in CAM, and their results were similar to those presented in this article. As far as the second mollusc, CN is concerned, the norms for AST, ALT and amylase activity, urea and triglycerides are published for the first time, and will surely serve as the basis for further research. Our own research included the determination of the normal AST: ALT ratio for both snail species. This indicator is useful and commonly used in the assessment and monitoring of liver damage in vertebrates (*Nyblom 2006*), and therefore it seems it may find application also in the evaluation of the hepatopancreas function in snails.

The experiments confirmed that administering large doses of doxycycline to snails may lead to high workload on the hepatopancreas, which results in higher activity of AST and ALT in the haemolymph of the experimental animals. The choice of the antibiotic was not accidental, but based on the fact that doxycycline is commonly, and informally used in helicultures (oral data). The hepatotoxicity of this antimicrobial in vertebrates is confirmed (Heaton et al. 2007). In our own

research, it was observed that the activity of AST and ALT in the haemolymph grew together with doxycyline administration time. Also the AST/ALT ratio changed in a way characteristic for mammals with liver damage (*Johnston 1999*).

Establishing the physiological norms of chosen biochemical parameters of snail haemolymph is important from the perspective of experimental research. The knowledge of physiological ranges of chosen biochemical parameters enables the monitoring of the health condition of snails (Tunholi-Alves et al. 2015), which is important for the use of molluscs as experimental models, especially in the light of current restrictions on the use of vertebrates in research.

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