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Common Carp (*Cyprinus carpio*) Blood Cells in Hayem's Solution – Stable or Not?

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Short communication

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A haematological analysis is commonly used in studies concerning fish, e.g. to evaluate the influence of various environmental factors on the physiological state of a population. Furthermore, a manual haematology is still used for conducting fish blood analyses. The aim of the current study was to determine how long after the procedure of blood collection in common carp (*Cyprinus carpio*) the samples diluted with Hayem's solution would provide results for the red blood cell and white blood cell counts with no significant changes. The blood was taken from 15 healthy individuals and diluted with Hayem's solution immediately after the collection procedure. Next, an erythrocyte count and leukocyte count were performed. The counts were repeated after 1, 2, 3, 5, 9, 16 and 23 days. The results of the current study indicate that the erythrocyte count should be performed within a maximum of 16 days (or better within 9 days) after the sample preparation, while the leukocyte count should be conducted within 2 days.

Key words: fish, erythrocyte, leukocyte, blood diluent, manual method.

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A blood analysis is an important element in the evaluation of a fish's physiological state, health and welfare (FAZIO 2019; WITESKA *et al.* 2022). It is also commonly used to assess an influence of various endogenous and exogenous factors on fish organisms (AHMED *et al.* 2020). Unfortunately, automatic analysers are rarely used in fish haematology (e.g. FAZIO *et al.* 2013; 2015; 2017; 2019; 2022). Fish haematologists still perform laborious and time-consuming analyses of red and white blood cells using a manual method (e.g. CHEN *et al.* 2019; CIEPLIŃSKI *et al.* 2019; ŁUSZCZEK-TROJNAR *et al.* 2019; KONDERA *et al.*

2020; BOJARSKI *et al.* 2021; GHAFARIFARSANI e*t al.* 2022; TOSIN *et al.* 2022). Blood cell counts are performed using a haemocytometer (e.g. Bürker chamber) and a light microscope. Before the analysis, the fish blood is routinely diluted with a special liquid, e.g. Natt-Herrick's or Hayem's solution. Sometimes the analysis of the erythrocyte (RBC) and leukocyte (WBC) counts takes a long time, especially in the case of large experiments which generate a large number of samples. However, to our knowledge, it has not yet been investigated how long fish blood cells diluted with Hayem's solution will remain stable. Thus, the aim of the current study was to determine how much time after the procedure of common carp (*Cyprinus carpio*) blood collection the samples diluted with Hayem's solution would provide results for the RBC and WBC counts with no significant changes.

Material and Methods

The experiment was conducted with the approval of the Local Ethics Committee for Animal Testing at the Medical University of Silesia in Katowice (Resolution No. 78/2021). In the current study, common carp (*Cyprinus carpio* L.) was used for the sample collection, since this species is a widely used model in scientific studies (WITESKA *et al.* 2022). The blood was collected from 15 clinically healthy fish (59.87 ± 8.02 g; 15.13 ± 0.55 cm). They were kept with other common carp individuals of a similar size and age, in a flow-through glass aquarium with a volume of 450 litres (with a total density about 30 fish per tank). The fish were kept in the aquarium for several months before the beginning of the experiment. They were fed daily with a standard feed intended for carp (1% of their body weight). The water physicochemical parameters (measured during the day of collecting the blood samples) were as follows: pH 7.4, total hardness 7°n, carbonate hardness 3°n, ammonia 0 mg/l, nitrites 0 mg/l, nitrates 10 mg/l, oxygen concentration 8.70 mg/l and temperature 17.8°C. Except for the temperature and oxygen concentration (which were determined using the Oxi 320 WTW oximeter) the values of the water physicochemical parameters were measured with aquaristical kits produced by the Zoolek company (Łódź, Poland).

The blood was taken from the caudal vein (*vena caudalis*) with previously heparinised plastic syringes and collected into heparinised Eppendorf tubes by an experienced haematologist without the use of anaesthetics, as it is known that anaesthetics can affect the haematological parameters (BISHKOUL *et al.* 2015; WITESKA *et al.* 2017a). According to WITESKA *et al*. (2022), the use of anaesthesia should be considered in fish that are highly susceptible or inevitably exposed to stress. However, in our practice we have observed that the use of anaesthetics is sometimes more stressful for common carp than a quick blood collection without the addition of pharmaceuticals. The blood sampling from each individual took approximately 2 minutes (in total). After the collection, the blood was immediately diluted 100 times with Hayem's solution provided by the Chempur company (Piekary Śląskie, Poland). Hayem's solution is commonly used in fish haematology (WITESKA *et al.* 2022). Next, the RBC count and WBC count were performed with a Bürker haemocytometer and a Delta Optical Evolution 300 microscope, using a 200x magnification for the erythrocyte analysis and a 400x magnification for the leukocyte count. The red blood cells were counted in an area of 0.2 mm^2, while the white blood cells were counted in an area of 2 mm^2. Next, the RBC and WBC counts were calculated using standard formulas (BOMSKI, 1983). The samples (diluted blood) were kept in a fridge (+4°C). The counting was then repeated 1, 2, 3, 5, 9, 16 and 23 days after the first analysis. Each parameter was determined by the same person during the experiment, and only experienced haematologists were involved in the analyses. Initially, we intended to finish the experiment after 5 days (which is enough time to finish a haematological analysis after sampling) but we noticed that the erythrocytes were very stable. For that reason, we decided to extend the duration of the experiment.

The compliance of the RBC and WBC results with the normal distribution was tested by the Shapiro-Wilk test. As the Shapiro-Wilk test did not reject the assumption that the variables studied came from a normal distribution (in all cases except one), a one-way ANOVA with repeated measures followed by a series of paired t-tests with a Bonferroni correction was applied. Only the results of the pairwise comparison between the first count and each other count were taken into consideration. The calculations were carried out using R free software (The R Foundation for Statistical Computing, version 4.1.2). The tables contain data expressed as the mean ±SD and adjusted p-values using a Bonferroni correction. For a single test, the p-value was the lowest level of significance at which the test rejected the null hypothesis. For the series of comparisons (t-tests), so-called Bonferroni corrections were applied. For each comparison, the adjusted p-value was the result of the multiplication of the p-value and the number of comparisons (if the result of the multiplication was lower of equal to 1) or the adjusted p-value was 1 (if the result of the multiplication was greater than 1). The significance level of each test was equal to 0.05.

Results

The RBC value obtained from the count performed on the day of the blood collection differed statistically significantly from none of the values obtained 1, 2, 3, 5, 9 and 16 days after the blood sampling. The first

statistically significant difference in the RBC values was observed 23 days after the blood collection (for the values of the RBC count reported as the mean ±SD, see Table 1).

The WBC value obtained from the count performed on the day of the blood collection differed statistically significantly from none of the values obtained 1 and 2 days after the blood sampling. The first statistically significant difference in the WBC values was observed 3 days after the blood collection. There were also significant differences between the result obtained from the count performed on the day of the blood sampling and each result obtained 5, 9, 16 and 23 days after the blood collection (for the values of the WBC count reported as the mean ±SD, see Table 2).

Table 1

Red blood cell count (mean ± SD) and p-values of the pairwise comparison carried out using a series of t-tests with Bonferroni corrections ($\alpha = 0.05$, n = 15)

Count No.	RBC count [per µl]	p-value
1st (after 0 days)	984,333 ± 227,783	not applicable
2nd (after 1 day)	876,333 ± 125,206	p = 1.000
3rd (after 2 days)	914,333 ± 126,235	p = 1.000
4th (after 3 days)	886,333 ± 149,433	p = 1.000
5th (after 5 days)	861,667 ± 117,286	p = 1.000
6th (after 9 days)	872,000 ± 103,644	p = 1.000
7th (after 16 days)	762,000 ± 156,214	p = 0.370
8th (after 23 days)	658,333 ± 130,037	p = 0.007

Table 2

White blood cell count (mean ± SD) and p-values of the pairwise comparison carried out using a series of t-tests with Bonferroni corrections ($\alpha = 0.05$, n = 15)

Count No.	WBC count [per µl]	p-value
1st (after 0 days)	18,967 ± 3,691	not applicable
2nd (after 1 day)	19,000 ± 4,049	p = 1.000
3rd (after 2 days)	16,167 ± 3,735	p = 0.459
4th (after 3 days)	14,633 ± 4,125	p = 0.034
5th (after 5 days)	13,300 ± 2,815	p = 0.001
6th (after 9 days)	13,367 ± 2,574	p = 0.000
7th (after 16 days)	11,867 ± 2,482	p = 0.000
8th (after 23 days)	8,400 ± 2,593	p = 0.000

Discussion

The values of the haematological parameters obtained in an experiment are influenced by the method of blood collection, the way of handling the samples, as well as the type and the method of conducting the analyses. BOJARSKI *et al.* (2018) compared the haematological values obtained from the cardiac and venous blood of common carp (*C. carpio*), and demonstrated that the haematocrit, haemoglobin concentration and erythrocyte count were higher in the venous samples in comparison to the cardiac ones. The study performed by BOJARSKI *et al.* (2021) showed that the RBC count was higher in the caudal vein blood, while the haematocrit value, mean corpuscular volume, mean corpuscular haemoglobin, total protein concentration and the magnesium concentration were higher in the cardiac blood samples of the same fish species. FAGGIO *et al.* (2013) assessed the effect of the storage of mullet (*Mugil cephalus*) whole (undiluted) blood and observed a significant effect of the sampling time on the haemoglobin concentration, mean corpuscular haemoglobin, mean corpuscular haemoglobin concentration, white blood cell count and the thrombocyte count. WITESKA *et al*. (2017b) demonstrated that the storage of common carp (*C. carpio*) whole (undiluted) blood may affect the haematological results. It was shown that storage for 2 h at 22°C resulted in a decrease of the erythrocyte, leukocyte and thrombocyte counts, while storage at 4°C led to an increase of the mean corpuscular haemoglobin and mean corpuscular haemoglobin concentration after 24 h. LUGOWSKA *et al.* (2017) conducted an experiment in which they considered the influence of the type of diluent, method of counting (direct vs. indirect) as well as the influence of the person counting the cells on the white blood cell count from the same common carp (*C. carpio*) group. They showed that the WBC count was affected by the person and method of counting, but not by the type of diluent. However, to our knowledge, it has so far not been determined how long blood samples diluted with a dedicated diluent (e.g. Hayem's solution) and stored in appropriate conditions (+4°C) can be used for an analysis of the RBC and WBC counts, without a significant change in the results.

The results obtained in this study indicate that the storage of fish blood diluted with Hayem's solution may lead to a significant decrease in the values of the WBC count and RBC count. The stability of the blood cells of common carp clearly depended on their type. The current results suggest that the RBC count should be determined within a maximum of 16 days (or preferably within 9 days) after the blood collection and dilution. However, the leukocytes turned out to be much less stable, as our results suggested that the WBC count should be determined within 2 days.

In the current paper, we demonstrated differences in the stability of erythrocytes and leukocytes diluted

with Hayem's solution over time. However, we did not study the mechanisms of the observed phenomena, which could be the subject of future studies.

In the present study we only tested one blood diluent, i.e. Hayem's solution, as we use this medium routinely. However, according to WITESKA *et al.* (2022), various diluting media are applied in fish haematology (e.g. Natt-Herrick's solution, Dacie's solution). Thus, we recommend testing the stability of fish blood cells in various diluents through further research.

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Author Contributions

Research concept and design: B.B.; Collection and/or assembly of the data: B.B., D.M., A.H., L.S., B.S., M.C.-G.; Data analysis and interpretation: B.B., D.M., A.H., L.S., B.S., M.C.-G.; Writing the article: B.B., D.M., A.H., L.S., B.S., M.C.-G.; Critical revision of the article: B.B., D.M., A.H., L.S., B.S., M.C.-G.; Final approval of the article: B.B., D.M., A.H., L.S., B.S., M.C.-G.

Conflict of Interest

The authors declare no conflict of interest in this study.

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