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Stability of haematological parameters in stored blood samples of rainbow trout *Oncorhynchus mykiss* (Walbaum, 1792)

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ABSTRACT: The aim of this study was to investigate the influence of storage time at +4 °C on haematological indicators in rainbow trout *Oncorhynchus mykiss* (Walbaum, 1792). For this purpose, 60 Italian trout were evaluated and red blood cells, white blood cells, thrombocyte count, haematocrit, haemoglobin concentration, mean corpuscular volume, mean corpuscular haemoglobin and mean corpuscular haemoglobin concentration were determined. After blood collection (T_0), all samples were immediately analysed using an automatic method to assess the haematological parameters and then divided into two different aliquots and stored at +4 °C. The first aliquot was refrigerated for 168 h (T_1), the second one for 336 h (T_2). Statistical analysis (one-way repeated-measures ANOVA) showed a significant effect of storage time ($P < 0.05$) on red blood cells, thrombocyte count, haematocrit, haemoglobin concentration, mean corpuscular haemoglobin and mean corpuscular haemoglobin concentration. The obtained data expand our knowledge of haematological evaluation techniques and could contribute to the development of an appropriate method for haematological analysis that is suitable for the evaluation of the health status of wild and farmed fish.

Keywords: fish farms; blood parameters; refrigeration; storage time

List of abbreviations

Hb = haemoglobin concentration, **Hct** = haematocrit, **MCH** = mean corpuscular haemoglobin, **MCHC** = mean corpuscular haemoglobin concentration, **MCV** = mean corpuscular volume, **RBCs** = red blood cells, **TC** = thrombocyte count, **WBCs** = white blood cells

The study of haematological parameters in fish is an important instrument for the evaluation of their physiological status, immune system and possible pathological changes (Ballarin et al. 2004; Tavares-Dias and Moraes 2004; Wells et al. 2005; Tavares-Dias and Moraes 2007; Micale et al. 2010). The number and size of red blood cells, haematocrit and haemoglobin concentrations, all indicate the oxygen-carrying capacity of blood (Tavares-Dias and Moraes 2004; Wells et al. 2005); together with white blood cells, these parameters are also indicators of toxicity and could be potentially employed for environmental monitoring and toxicity studies in aquatic animals (Sancho et al. 2000; Barcellos et al. 2003).

One of the major difficulties in assessing the health status of a natural population of fish is the lack of reliable references values from healthy specimens of a given species (Nicula et al. 2010; Fazio et al. 2012; Fazio et al. 2015). Many authors have focused their studies on haematological parameters because they can provide important diagnostic information if standardised reference values are established (Huffman et al. 1997; Clauss et al. 2008; Buscaino et al. 2010).

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Haematological analyses must be carried out immediately after blood collection; or, if this is not possible, samples should be refrigerated to minimise artifactual changes (Wood et al. 1999). Previous studies have shown that storage at +4 °C may stabilise samples for periods of 24–72 h before blood cell counts are performed (Buttarello 2004).

Sample stability is defined as the capability of a sample to retain the initial value of a measured quantity for a defined period within specific limits when stored under defined conditions (Guder 1999).

In fish, it is recommended to perform haematological determinations immediately upon collection; however, often this is not possible, especially when blood samples are collected from remote aquaculture farms. Only a few studies investigating the stability of haematological parameters in fish have been published to date (Fazio et al. 2012; Faggio et al. 2013). It is well known that the handling of blood samples, as well as the method of storage, can significantly influence the results of haematological determinations and consequently, these factors can result in blood samples yielding misleading results. Therefore, it is important to identify the optimal timing for the analysis of blood parameters in fish. With this aim in mind, this study was carried out to investigate whether the storage of blood samples at 4 °C for timeframes of varying duration (0, 168, and 366 h), influences the results of routine haematological analysis in rainbow trout *Oncorhynchus mykiss* (Walbaum, 1792). These results contribute to our understanding of whether changes caused by delayed analysis result in clinically relevant differences, and help in establishing guidelines for acceptable storage times in this species.

MATERIAL AND METHODS

A total of 60 rainbow trout (*Oncorhyncus mykiss*) provided by a commercial farm in Palazzolo Acreide (Siracusa, Italy) were investigated in this study. The research was carried out in November 2015. All farmed fish were judged to be in excellent health (on the basis of external examinations for any signs of abnormalities or infestation) and were subjected to a natural day/night cycle (11L/13D). The fish were fed a commercial dry food (crude protein 46%; crude fat 20%; ash 10%; fiber 1.5%) distributed twice daily seven days a week.

The physical and chemical characteristics of water were recorded on the farm. Temperature, salinity, pH and dissolved oxygen were measured using a handheld multiparameter instrument (model YSI 556 MPS – Ohio, USA) and their values are as follows: temperature 16.60 ± 0.26 °C, salinity 0.30 ± 0.06 ppt, pH 8.23 ± 0.09 and dissolved oxygen 7.33 ± 0.09 mg/l.

All fish were randomly captured in the same tank for evaluation of biometric and haematological parameters. After capture, the fish were anaesthetised prior to blood sampling using MS222 at a concentration of 0.7 g/l. Immediately after anaesthetisation the fish were individually weighed using a balance (Kern 440-49 N, Germany) and their total length was recorded using an ictiometer (Scubla SNC, 600 mm, Italy). The biometric data of the rainbow trout (*Oncorhynchus mykiss*) were as follows: weight 507.40 ± 34.72 and length 32.90 ± 0.64 cm.

The condition factor was also calculated as follows:

$$K = W \times 100/L^3$$

where: K = condition factor; W = weight of the fish in grams; L = length of the fish in centimetres

For salmonids, condition factor values usually fall in the range of 0.8 to 2.0 (Davis and Lebourdais 2007). The condition factor value that we obtained was 1.40 ± 0.03.

Blood samples were collected between 8.00 a.m. and 10.00 a.m. and were obtained from puncture of the caudal vein using an 18 G × 1½ syringe and transferred into microtubes (Miniplast 0.6 ml; LP Italiana Spa, Milano) containing ethylenediaminetetraacetic acid (ratio 1.26 mg/0.6 ml) as the anticoagulant agent for the assessment of haematological parameters.

The time between capture and blood sampling was less than 3 min. All samples were analysed immediately (T_0) by an automatic method using the HeCoVet C (SEAC, Florence, Italy) blood cell counter, which has previously been validated and used in this and other species with manual and automatic systems (Faggio et al. 2013; Fazio et al. 2016).

The samples were then divided into two different aliquots and stored (refrigeration temperature +4 °C) for different periods of time (168 and 366 h) in order to evaluate the effect of storage time on haematological parameters.

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Analytical procedures were performed to determine the following parameters: red blood cells (RBCs), haematocrit (Hct), haemoglobin (Hb), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), white blood cells (WBCs) and thrombocyte count (TC).

All haematological analyses were performed in triplicate by the same operator with the same instrument under the same conditions, and in a short period of time.

All experimental procedures were carried out in accordance with European legislation regarding the protection of animals used for scientific purposes (European Directive 2010/63).

Statistical analysis. Analytical data, represented as mean ± SD, are the averages of three analyses carried out by the same operator. Samples exhibited parallel displacement to the standard curve. The overall intra-assay coefficient of variation was less than 9%.

Data were normally distributed ($P > 0.05$; Kolmogorov-Smirnov test). The influence of sampling time on haematological parameters evaluated with the automatic method was assessed by one-way analysis of variance (ANOVA) for repeated measures. P-values < 0.05 were considered statistically significant. Bonferroni's multiple comparison test was applied for post hoc comparison. Data were analysed using GraphPad Prism v. 5.00 statistical software (Graphpad Software Ltd., USA, 2003).

RESULTS

Statistical results for the evaluated haematological parameters in farmed rainbow trout (*Oncorhynchus mykiss*) after storage for different periods of time (0, 168, and 366 h after collection) are reported in Table 1. One-way repeated-measures ANOVA showed a statistically significant effect of storage time on the measured parameters. RBCs ($P < 0.0001$), Hct ($P < 0.0001$), MCH ($P < 0.0001$), and MCHC ($P < 0.0001$) decreased at 168 and 366 h with respect to time point 0. TC ($P = 0.0001$) and Hb ($P < 0.0001$) decreased at 366 h with respect to time point 0.

Table 1. Evaluated haematological parameters (mean ± SEM) in farmed rainbow trout (*Oncorhynchus mykiss*). Means without the same alphabetical characters for the same parameters indicate statistical differences ($P < 0.05$)

Parameters	Storage time		
	T_0	T_1	T_2
RBCs ($\times 10^6$/μl)	1.55 ± 0.03^a	1.40 ± 0.02^b	1.47 ± 0.03^b
WBCs ($\times 10^3$/μl)	20.17 ± 0.22^a	20.13 ± 0.17^a	20.22 ± 0.17^a
TC ($\times 10^3$/μl)	59.50 ± 5.37^a	50.85 ± 2.12^{ab}	43.95 ± 2.56^b
Hct (%)	29.66 ± 0.79^a	27.16 ± 0.69^b	26.63 ± 0.77^b
Hb (mmol/l)	1.63 ± 0.08^a	1.60 ± 0.08^{ab}	1.48 ± 0.05^b
MCV (fl)	191.40 ± 2.50^a	189.10 ± 1.65^a	184.90 ± 2.28^a
MCH (pg/cel)	74.75 ± 2.69^a	66.18 ± 2.25^b	65.03 ± 1.66^b
MCHC (%)	39.57 ± 1.43^a	35.24 ± 0.96^b	34.52 ± 1.02^b

Hb = haemoglobin concentration, Hct = haematocrit, MCH = mean corpuscular haemoglobin, MCHC = mean corpuscular haemoglobin concentration, MCV = mean corpuscular volume, RBCs = red blood cells, TC = thrombocyte count, WBCs = white blood cells

DISCUSSION

Evaluation of haematological parameters has been widely applied in aquaculture systems to determine the health status of fish; therefore, it is necessary to evaluate the effects of time from collection on the outcome of these results. Our results show that storage time (168 and 366 h) at +4 °C influences some of haematological parameters studied. In particular, the statistically significant decrease in RBCs, TC, Hct, Hb, MCH and MCHC observed is probably due to the damage to erythrocytes caused by the long-term storage, which was then also reflected in the altered Hb values. Blood samples kept for long periods of time exhibit storage-related degenerative changes that occur in the RBCs and lead to a widening of the "pores" on the surface of the RBCs, permitting the ingress of water into the cells. Therefore, the decrease in Hb during storage is directly related to the significant changes in MCH and MCHC values. This is in contrast to what was previously observed in another species of fish (Fazio et al. 2012), in which RBC did not show statistically significant changes and Hb values significantly increased during the storage time.

Many studies have reported changes in several haematological parameters in stored and refriger-

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ated samples of horses (Clarke et al. 2002), bovines, pigs (Bluel et al. 2002; Ihedioha and Onwubuche 2007), goats and rats (Ihedioha and Onwubuche 2007) and chickens (Ihedioha et al. 2008). However, in fish, only a few studies were aimed at determining the changes in the haematological profile in response to different storage times. Our results are not in agreement with previous data that showed a decrease in the RBC profile to occur after 168 h of storage at 4 °C in Gilthead sea bream *Sparus aurata* and Flathead grey mullet *M. cephalus*.

Therefore, it would seem that trout blood is particularly sensitive to storage time compared to that of other species of fish. Moreover, during the storage of the sample a reduction of TC was also evident. This reduction, starting at 168 h and persisting until 366 h, could be related to the particular sensitivity of the thrombocytes in the sample to undergo degeneration. Further, these observed changes could be due to the presence of platelet aggregates induced by the long-term storage.

Among the different types of blood cells, WBCs seem to be particularly resistant to storage time. In fact, no changes were observed in response experimental storage times of different durations (168 and 366 h). These results are in accordance with previous research carried out in mullet (Faggio et al. 2013) and could be related to species-specific factors that promote particular resistance of these cells to refrigeration temperatures.

Based on our results, we conclude that trout blood samples stored for up to 366 h at 4 °C do show changes in some haematological parameters. These results, together with those reported in other species (Fazio et al. 2012; Faggio et al. 2013), confirm that long-term storage (166 h and above) influences the analysis of haematological parameters in fish. To avoid artefacts, samples should be assessed within 6 h after collection if stored at +4 °C (Faggio et al. 2013).

The data obtained in this study expand our knowledge of haematological evaluation techniques in fish. Further, it is our hope that they will contribute to the development of appropriate methods of haematological analysis for evaluation of the health status of wild and farmed fish.

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