



Effect of Three Different Anticoagulants and Storage Time on Haematological Parameters of *Mugil cephalus* (Linnaeus, 1758)

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Abstract

Anticoagulants and storage time's effect on hemogram and differential leukocyte count in 26 *Mugil cephalus* was investigated. After blood collection, samples were put, in three microtubes containing respectively: ethylenediamine tetraacetic acid (EDTA), heparin, sodium citrate. Analysis were assessed immediately (T0) and 24 h (refrigerated at +4°C) after collection (T24). Statistical analysis showed: significant higher levels of hematocrit (Hct) and hemoglobin (Hb) and lower levels of thrombocytes count (TC) in EDTA ($P < 0.001$) than heparin and sodium citrate treated samples at T0 and T24; white blood cell (WBC) and Lymphocytes values were lower ($P < 0.05$) in sodium citrate than with EDTA and heparin treated samples at T24, while Monocytes levels were higher ($P < 0.05$) in sodium citrate than EDTA and heparin treated samples at T24. Statistical analysis showed also a significant effect of storage time ($P < 0.01$) on TC, Monocytes, Neutrophils, mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC) which statistically increase from T0 to T24, while WBC and Lymphocytes statistically decrease from T0 to T24. These findings demonstrate that EDTA showed higher reliability as anticoagulant for this fish and that hematological determinations can be assessed within 24h hours from collection, because storage time modifies analysis results.

Keywords: Fish, EDTA, heparin, sodium citrate, hematology.

Introduction

Hematology is used as an index of fish health status in a several number of fish species to detect physiological changes following different stress condition like exposure to pollutant, diseases, metals, hypoxia (Walencik and Witeska, 2007).

Hematological parameters are closely related to the response of the animal to the environment, an indication that the environment where fishes live could exert some influence on blood characteristics (Gabriel *et al.*, 2004).

Interpretation of fish hematological data is quite difficult due to internal and external variation. It is well known that blood sampling, laboratory techniques, storage and handling of blood samples between the time of blood collection and the time of evaluation in the laboratory could strongly influence the results obtained from hematologic determination (Nemeth *et al.*, 2009; Clark *et al.*, 2011). Thus it is routinely recommended that hematologic determinations on blood samples are carried out immediately after blood collection, and if not possible, the samples should be refrigerated until

determination to minimize artifactual changes (Wood *et al.*, 1999).

Moreover, as fish blood tends to clot rapidly and clotting becomes faster when it is warm or is under stress condition related to experimental procedures (Korcock *et al.*, 1988), the use of anticoagulants is necessary to obtain reliable results of blood analyses. The diagnostic techniques applied in fish hematology are usually adapted methods developed in human hematology. This concerns also anticoagulants and their concentrations (Blaxhall, 1972). Anticoagulants most commonly applied in human clinical hematology include sodium and potassium salts of ethylenediamine tetra-acetic acid (EDTA), heparin, and sodium citrate. The analysis of several papers concerning hematological studies of various fish species published over 1960–2003 (list available from authors), revealed that heparin is the most commonly used anticoagulant (80%), followed by EDTA (12%), while sodium citrate are rarely applied (4% each) (Walencik and Witeska, 2007).

Heparin is a natural endogenous anticoagulant agent acting both in blood vessels *in vivo*, and *ex vivo* when added to a blood sample. Heparin inhibits

conversion of prothrombin into active thrombin, and thus prevents conversion of fibrinogen into fibrin. The mechanism of EDTA and sodium citrate anticoagulant action is based on inhibition of thrombocyte aggregation and various reactions of hemostatic cascade due to chelation of free Ca^{2+} ions (Witeska and Wargocka, 2011), important cofactors of several serine proteases of the blood coagulation cascade.

Some authors consider heparin the most suitable anticoagulant in fish hematology (Walencik and Witeska, 2007; Svobodova *et al.*, 1991; Ekanem *et al.*, 2012), however, according to the others EDTA is an anticoagulant of choice because it preserves blood components for a long time and is very effective in prevention blood clotting (Blaxhall, 1972; Blaxhall *et al.*, 1973).

However, there is very little evidence of the effects of anticoagulants on various blood parameters of various animals. In human hematology, it is assumed that heparin should not be used for blood smears due to staining background blue, or for WBC count due to clotting leukocytes, while EDTA is inappropriate for evaluation of erythrocyte osmotic fragility, and its excess may cause damage to the blood cells (Marianska *et al.*, 2003). On the other hand, EDTA shows a tendency to increase hematocrit in fish, may induce hemolysis in some species due to its adverse effect on cell membranes, causes deformation of erythrocytes and a decrease in fish leukocyte viability (Walencik and Witeska, 2007).

The effects of various types of anticoagulants in hematology were studied in various fish species (Walencik and Witeska, 2007; Witeska and Wargocka, 2011; Ekanem *et al.*, 2012; Adeyemo *et al.*, 2009) but no information's for *M. cephalus* hematology were found.

The aim of the present study was to compare the values of hematological parameters and differential leukocyte count measured in *M. cephalus* collected on three anticoagulants: EDTA, heparin and sodium citrate.

In addition we wanted to assess whether and how these three anticoagulants affect hematological parameters and differential leukocyte count in two different storage conditions, immediately after blood collection (T0) and 24 h at $+4^{\circ}\text{C}$ after blood collection (T24), to evaluate their usefulness for routine fish blood analysis.

Materials and Methods

Experimental Procedures

Twenty-six adult male mullets (*M. cephalus*), caught from Faro Lake (Sicily, Italy), were used. The animals, weighing 94.98 ± 16.70 g and 30.22 ± 3.32 cm in total length (Mean Values \pm Standard Deviation), were considered as healthy on the basis of an external examination for any signs of abnormalities or

infestation. On the basis of their weight and length, all fish were considered sexually mature and with age between 2 and 4 years (McDonough *et al.*, 2005). The fish, transported from the site of capture to the laboratory of the "Sicilian Centre for Experimental Fish Pathology (CISS)", were acclimated before the experiment for 6 weeks in 800-l tanks with flowing freshwater (temperature, 22.4°C ; salinity, 33.4 ppm; pH 8.1) to restore the effects of capture, handling and transport. During the acclimatizing period, fish were kept under artificial photoperiod (12h Light and 12h Dark), and they were kept fasting for the first 3 days and successively they were fed to satiation twice daily with commercial floating pelleted feed (0.45 cm diameter). The proximate composition of the feed on wet basis was 8.9% moisture, 51.1% protein, 8.0% lipid and 11.0% ash. Fish were not fed 24 h prior the blood collection. Prior to blood sampling fish were anesthetized using 2-Phenoxyethanol (99%, MERCK, Whitehouse Station, NJ, USA) at the concentration of 400 mg/l.

Blood samples were obtained by puncturing the caudal vein using a 20 x 1/2-gauge syringe. Each blood sample was then put, triplicate, in microtubes containing three different anticoagulants: ethylenediaminetetraacetic acid (EDTA) (ratio, 1.26 mg/0.6 mL), heparin (50 IU/ml) (Witeska and Wargocka, 2011) and sodium citrate (0.3 mg/ml) (Walencik and Witeska, 2007). The time between capture and blood sampling was less than 3 min. On one aliquot of each sample hematological analysis and manual differential leukocyte counts were carried out immediately (T0). The second aliquot of each sample was stored at $+4^{\circ}\text{C}$ to evaluate the effect of storage time at 24 h after collection (T24) on hematological parameters and manual differential leukocyte counts.

Automatic Hematological Analysis

All samples were analyzed in triplicate by the same operator immediately after collection. The samples exhibited parallel displacement to the standard curve. The overall intra-assay coefficient of variation was $<5\%$. The samples, stored under refrigeration, were subjected to the same analytical procedure repeated 0 and 24 h after collection.

A total of 104 analytical measurements were made to determine red blood cell count (RBC), hematocrit (Hct), hemoglobin (Hb), white blood cell count (WBC), thrombocyte count (TC), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC).

The automatic analysis was performed using the blood cell counter HeCo Vet C (SEAC, Florence, Italy) that is already in use in the veterinary field. In fact, this automatic procedure has been previously used in *M. cephalus* and in other fish species (Fazio *et al.*, 2012a, 2012b, 2012c, 2013).

Before analyzing the samples, they were allowed

to equilibrate with room temperature (18°C) then gently mixed for 15 min. All measurements were carried out individually, and the other samples were kept at refrigeration temperature to avoid the interruption of the storage temperature.

Manual Differential Leukocyte Counts

To leukocyte identification and counting, a manual analysis was performed on all samples for each anticoagulants. After blood collection, duplicated blood smears were done for each fish. After air-drying, the slides were stained through "DIF-STAIN" kit (TITOLCHIMICA S.r.l., Rome, Italy). After washing the excess dye from the blood smears and air-drying, the slides were viewed under oil immersion at 100x with optical microscope (model B-150 - OPTICA, Microscopes, Italy). Approximately 200 cells were counted in random fields of view.

Protocols of fish and experimentation were reviewed and approved in accordance with the standards recommended by the *Guide for the Care and Use of Laboratory Animals* and Directive 63/2010/EU.

Statistical Analysis

The influence of different anticoagulants and storage time on haematological parameters and leukocyte identification and counting were assessed by two-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. All data were analyzed using the PRISM package (GraphPad Prism, Version 4.00, 1992-2003, Software Inc., San Diego, California).

Results

Mean Values \pm SD of studied haematological parameters and the percentage of leukocyte

identification and counting were reported in Table 1.

Two-way analysis of variance (ANOVA) for repeated measures showed statistical significant effects of different anticoagulants and storage time for some studied parameters.

In particular, as shown in Figure 1, statistical analysis showed significant effect of anticoagulants on Hct, Hb, TC (P<0.001), WBC, Lymphocytes and Monocytes (P<0.05).

In Figure 2, significant effects of storage time (T0 and T24) on MCH, MCHC, TC, WBC, Lymphocytes, Monocytes and Neutrophils (P<0.01) were showed.

Discussion

The choice of anticoagulant and storage time is of major importance when blood samples are to be used in laboratory analysis because, it is well known that blood parameters are likely influenced by these two independent variables (Ekanem *et al.*, 2012; Faggio *et al.*, 2013).

The three anticoagulants selected for this study have been successfully used in routine laboratory analysis. In particular EDTA is the anticoagulant recommended for full blood cell counts and white blood cell differential analysis by the NCCLS (National Committee for Clinical Laboratory Standards, 1996), principally for its cell preservation properties. Heparin is considered the most suitable anticoagulant in haematology but it is too expensive and it is known that it affects the staining properties of cells (Golanski *et al.*, 1996). Citrate is used as an anticoagulant primarily for coagulation studies.

Our results showed that some haematological parameters and differential leukocyte count were significantly affected by anticoagulants and storage time.

In particular, Hct and Hb values in EDTA were statistically higher compared with values in heparin and sodium citrate at T0 and T24.

Generally, high hematocrit and high Hb were

Table 1. Mean values \pm Standard Deviation (SD) of haematological parameters and percentage of leukocyte identification and counting obtained in 26 *Mugil cephalus*

Parameters	T0			T24		
	EDTA (M \pm SD)	Sodium Citrate (M \pm SD)	Heparin (M \pm SD)	EDTA (M \pm SD)	Sodium Citrate (M \pm SD)	Heparin (M \pm SD)
RBC (x10 ⁶ /μl)	2.55 \pm 0.41	2.26 \pm 0.76	2.34 \pm 0.91	2.30 \pm 0.65	2.13 \pm 0.63	2.28 \pm 0.67
Hct (%)	30.14 \pm 5.74	26.89 \pm 4.89	25.43 \pm 4.83	30.22 \pm 5.74	24.17 \pm 3.01	26.25 \pm 5.76
Hb (g/dl)	7.34 \pm 1.18	6.13 \pm 1.83	6.54 \pm 1.91	7.57 \pm 1.00	6.60 \pm 1.69	7.27 \pm 1.63
WBC (x10 ³ /μl)	8.02 \pm 2.14	7.95 \pm 3.08	8.03 \pm 3.26	7.25 \pm 2.05	5.08 \pm 2.26	7.20 \pm 2.99
Lymphocytes (%)	90.80 \pm 2.60	90.10 \pm 2.60	91.40 \pm 3.80	89.80 \pm 5.00	84.40 \pm 7.40	88.30 \pm 6.60
Monocytes (%)	2.50 \pm 1.00	2.90 \pm 2.10	2.90 \pm 2.80	3.30 \pm 1.90	6.00 \pm 3.90	4.00 \pm 2.60
Neutrophils (%)	6.70 \pm 2.30	7.00 \pm 2.40	5.70 \pm 2.30	6.90 \pm 3.30	9.20 \pm 3.90	7.30 \pm 4.50
Eosinophils (%)	0.00 \pm 0.02	0.00 \pm 0.00	0.00 \pm 0.20	0.00 \pm 0.20	0.10 \pm 0.30	0.20 \pm 0.50
Basophils (%)	0.00 \pm 0.00	0.00 \pm 0.00	0.00 \pm 0.20	0.00 \pm 0.20	0.30 \pm 0.30	0.20 \pm 0.50
TC (x10 ³ /μl)	22.36 \pm 3.87	24.59 \pm 3.65	24.99 \pm 3.14	26.25 \pm 3.48	29.86 \pm 2.92	28.65 \pm 3.39
MCV (fL)	119.84 \pm 22.66	130.67 \pm 44.23	117.93 \pm 32.27	147.95 \pm 69.98	122.10 \pm 34.86	121.38 \pm 37.17
MCH (pg)	28.89 \pm 1.88	27.88 \pm 5.16	28.99 \pm 3.74	38.39 \pm 16.54	33.23 \pm 10.69	34.22 \pm 12.53
MCHC (g/dl)	24.65 \pm 2.79	23.19 \pm 7.19	25.65 \pm 4.95	25.59 \pm 4.00	27.56 \pm 7.42	27.89 \pm 3.61

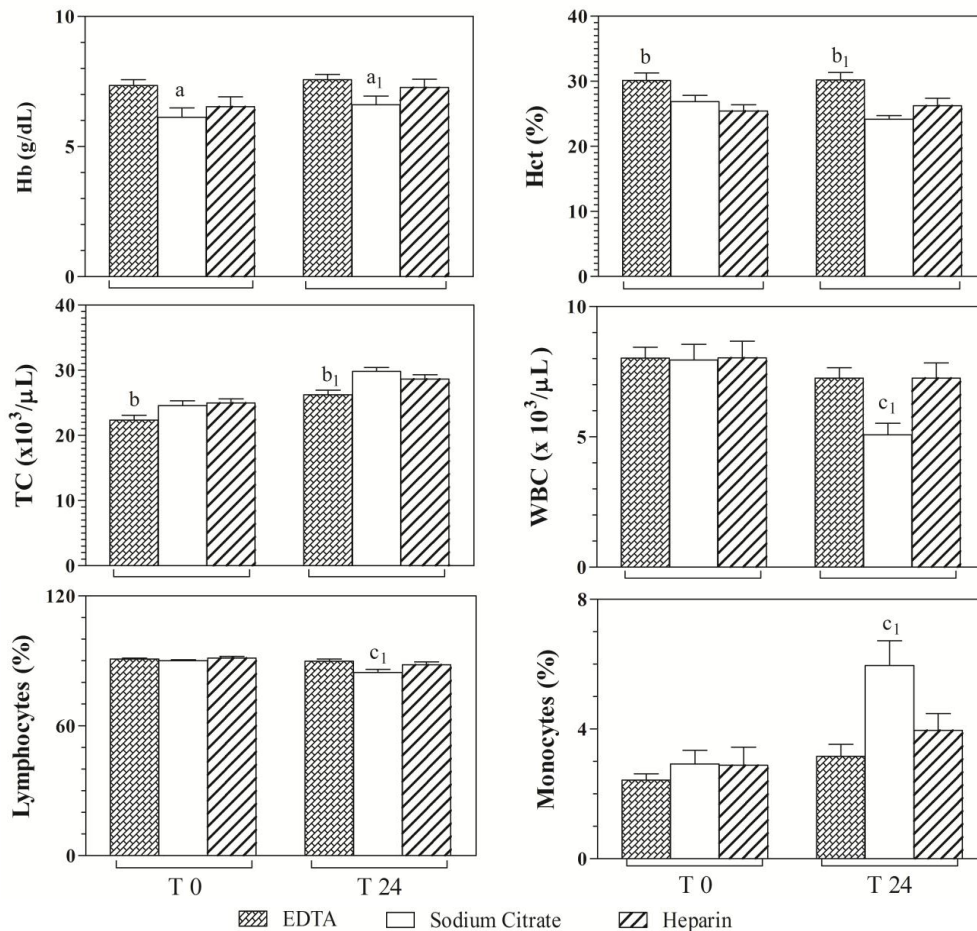


Figure 1. Significant effect of the three anticoagulants used (EDTA, Heparin and Sodium Citrate) on haematological parameters recorded in *M. cephalus* (n=26).

Significances: ^avs EDTA at T=; ^{a1}vs EDTA at T24; ^bvs Heparin and Sodium Citrate at T0; ^{b1}vs Heparin and Sodium Citrate at T24; ^{c1}vs Heparin and EDTA at T24.

related to erythrocyte swelling and hemolysis, respectively (Witeska and Wargocka, 2011). Erythrocyte swelling may be due to an increase in pCO₂ and acidification due to treatment with acidic EDTA salt (Smit *et al.*, 1977). Erythrocytes swelling in EDTA treated samples was also observed by Blaxhall (1973) and Korcock *et al.* (1988) in fish, and by Olsen *et al.* (2001) in mammal blood. In contrast, a decrease in hematocrit values in mammal blood samples with EDTA compared to heparinized blood was reported (Morris *et al.*, 2002), while EDTA did not affect Hct values in green iguana blood (Hanley *et al.*, 2004). Hemolysis in EDTA-anticoagulated samples was reported in rainbow trout and in common carp (Korcock *et al.*, 1988; Walencik and Witeska, 2007).

In the present study higher Hct and Hb values in EDTA respect to heparin and sodium citrate are directly related with higher RBC found in EDTA treated sample and indicate a better preservation of cells in EDTA treated samples.

WBCs difference were in agreement with statistically changes found in manual differential leukocyte count.

A possible reason that may be responsible for

the off-shoot of the values of white blood cells in the anticoagulated blood is the recognition of the presence of anticoagulants as foreign bodies within the cells thereby stimulating the production of more cells (WBC) to fight against them as self-defense.

In the literature, the data concerning the effects of anticoagulants on white blood cells are very scarce. Cells reaction of various animal species to anticoagulants may be different. Studies on several fish have found that blood cells were unaffected by EDTA, heparin or sodium citrate but both EDTA and citrate reduced white blood cell viability (Walencik and Witeska, 2007; Adeyemo *et al.*, 2009).

However differences are difficult to explain. It may result from different buffering capacity of blood or from different sensitivity of cell membranes to extracellular decalcification due to Ca²⁺ chelation by anticoagulant.

The analysis of the results showed that the storage time (T0 and T24) affect MCH, MCHC, TC, WBC, Lymphocytes, Monocytes and Neutrophils in EDTA, heparin and sodium citrate treated samples. RBC, Hb and MCV values showed a slight increase at T24 despite no significant change was found. Probably the long storage time involved a slight

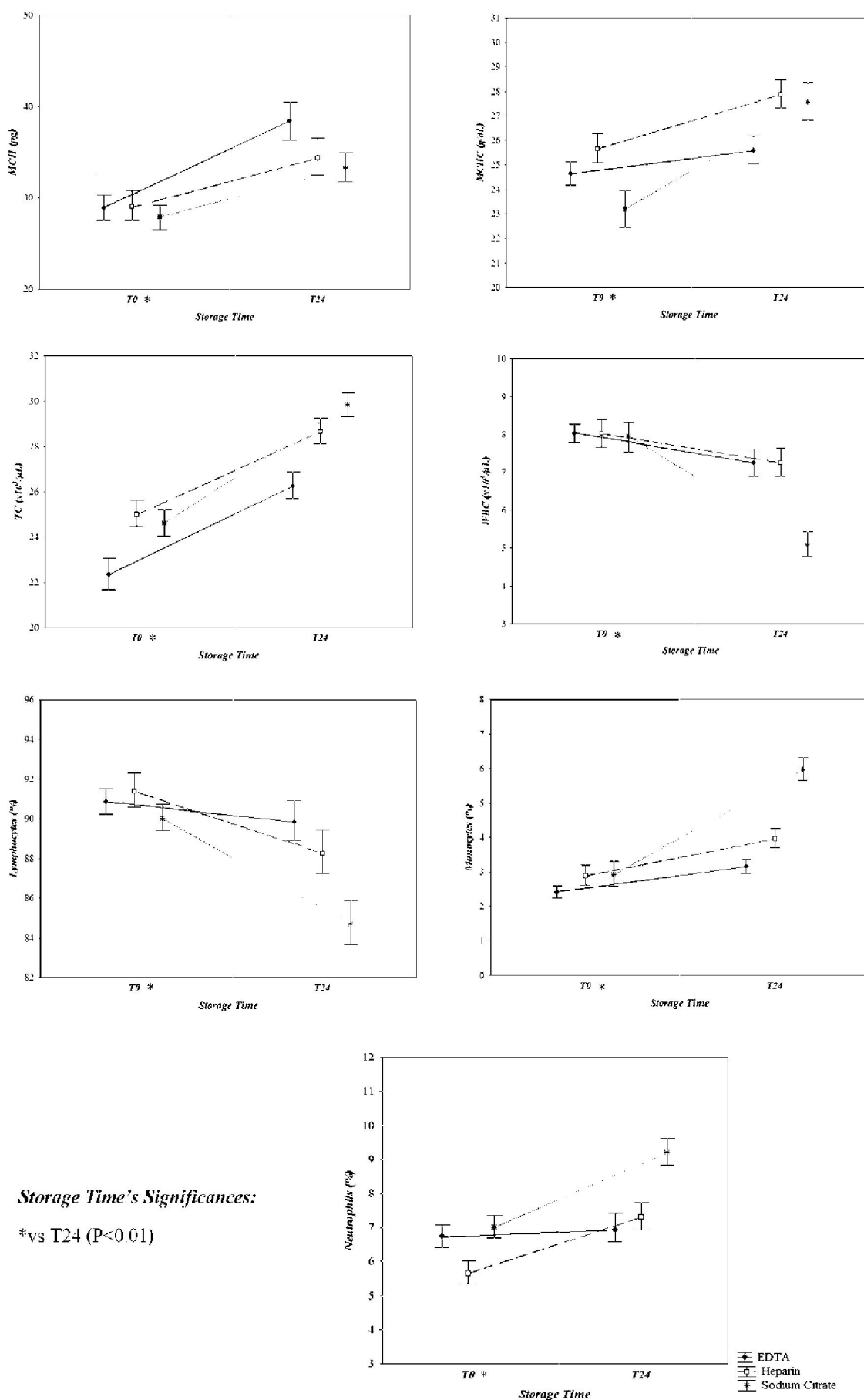


Figure 2. Significant effect of storage time (T0 and T24) on haematological parameters recorded in *M. cephalus* (n=26).

erythrocyte pain, which was reflected in the change of MCH and MCHC values that statistically increase from T0 to T24.

Similar modifications in *S. aurata* (Fazio et al., 2012b) and in our previous work on *M. cephalus* were observed (Faggio et al., 2013). Studies on human and equine blood samples reported an increase in volume of RBC stored for several hours both at room and refrigerator temperature (Clarke et al., 2002). In rainbow trout, it has been demonstrated that the viability of RBC may be compromised if blood is stored for longer than 96 h (Caldwell et al., 2006).

Hence, as shown by literature, the period during which the assessed parameters change significantly from baseline values varies from one species to the other. The changes occurred in TC, WBC, Lymphocytes, Monocytes and Neutrophils values with the increasing time of storage were not found in literature. The significant decrease in WBC and Lymphocytes could be attributed to cellular degeneration because of the time of storage, whereas the significant increase in TC, Monocytes and Neutrophils could be due to the presence of white cell nuclei that were wrongly added to the TC.

By the results of this study, it was found that haematological parameters and differential leukocyte count of *M. cephalus* were influenced by anticoagulants used and that, according to our previous study (Faggio et al., 2013), haematologic determinations should be assessed before the 24 hours after collection, because long-term storage modifies the results of the analysis.

All haematological parameters values obtained in EDTA, heparin and sodium citrate treated samples at T0 fall within values obtained in our previous study on *M. cephalus* (Faggio et al., 2013) and leukocytes differential count of the samples treated with the three anticoagulants were within fish reference range (Smith, 2007). Thus, haematologic determination and leukocyte differential count showed no cellular alteration depending on anticoagulants used. However parameters studied showed a higher reliability using EDTA as anticoagulant.

Comparison of literature data indicates that modifications of blood parameters induced by internal or/and external factors, such as anticoagulants and storage time, could represent a species-specific responses. Further studies designed specifically to investigate the impact of different anticoagulants and storage times on these parameters could be still needed in various fish species to validate an appropriate method for haematological analysis useful for the evaluation of the health status of animal living in captivity and in aquaculture.

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References

- Adeyemo, O.K., Okwilagwe, O.O. and Ajani, F. 2009. Comparative assessment of sodium EDTA and heparin as anticoagulants for the evaluation of haematological parameters in cultured and feral african catfish (*Clarias gariepinus*). *Brazilian Journal of Aquatic Science and Technology*, 13: 19-24. doi: 10.14210/bjast.v13n1.p19-24.
- Blaxhall, P.C. 1972. The haematological assessment of the health of freshwater fish. *Journal of Fish Biology*, 4: 593-604. doi: 10.1111/j.1095-8649.1972.tb05704.x.
- Blaxhall, P.C. and Daisley, K.W. 1973. Routine haematological methods for use with fish blood. *Journal of Fish Biology*, 5: 771-781. doi: 10.1111/j.1095-8649.1973.tb04510.x.
- Caldwell, S., Rummer, J.L. and Brauner, C.J. 2006. Blood sampling techniques and storage duration: effects on the presence and magnitude of the red blood cell b-adrenergic response in rainbow trout (*Oncorhynchus mykiss*). *Comparative Biochemistry and Physiology Part A*, 144: 188-195. doi: 10.1016/j.cbpa.2006.02.029.
- Clarke, P., Mogg, T.V., Tvedten, H.W. and Korcal, D. 2002. Artifactual changes in equine blood following storage, detected using the Advia 120 hematology analyzer. *Veterinary Clinical Pathology*, 31: 90-94. doi: 10.1111/j.1939-165X.2002.tb00286.x.
- Clark, T.D., Donaldson, M.R., Drenner, S.M., Hinch, S.G., Patterson, D.A., Hills, J., Ives, V., Carter, J.J., Cooke, S.J. and Farrell, A.P. 2011. The efficacy of field techniques for obtaining and storing blood samples from fishes. *Journal of Fish Biology*, 79: 1322-1333. doi: 10.1111/j.1095-8649.2011.03118.x.
- Faggio, C., Casella, S., Arfuso, F., Marafioti, S., Piccione, G. and Fazio, F. 2012. Effect of storage time on haematological parameters in mullet, *Mugil cephalus*. *Cell Biochemistry and Function*, 31: 412-416. doi: 10.1002/cbf.2915.
- Fazio, F., Satheeshkumar, P., Senthil Kumar, D., Faggio, C. and Piccione, G. 2012a. A comparative study of haematological and blood chemistry of Indian and Italian Grey Mullet (*Mugil cephalus* Linnaeus, 1758). *Herbert Open Access Journal Biology*, 5: 1-5. doi: 10.7243/2050-0874-1-5.
- Fazio, F., Filiciotto, F., Marafioti, S., Di Stefano, V., Assenza, A., Placenti, F., Buscaino, G., Piccione, G. and Mazzola, S. 2012b. Automatic analysis to assess haematological parameters in farmed gilthead sea bream (*Sparus aurata* Linnaeus, 1758). *Marine and Freshwater Behaviour and Physiology*, 45: 63-65. doi: 10.1080/10236244.2012.677559.
- Fazio, F., Marafioti, S., Torre, A., Sanfilippo, M., Panzera, M. and Faggio, C. 2013. Haematological and serum protein profiles of *Mugil cephalus*: effect of two different habitat. *Ichthyological Research*, 60: 36-42. doi: 10.1007/s10228-012-0303-1.
- Gabriel, U.U., Ezeri, G.N.O. and Opabunmi, O.O. 2004. Influence of sex, source, health status and acclimation on the haematology of *Clarias gariepinus* (Burch,

- 1822). African Journal of Biotechnology, 3: 463-467. doi: 10.5897/AJB2004.000-2090.
- Golanski, J., Pietrucha, T., Baj, Z., Greger, J. and Watala, C. 1996. Molecular insights into the anticoagulant-induced spontaneous activation of platelets in whole blood-various anticoagulants are not equal. Thrombosis Research, 83: 199-216. doi: 10.1016/0049-3848(96)00129-6.
- Hanley, C., Hernandez-Divers, S.J., Bush, S. and Latimer, K.S. 2004. Comparison of the effects of dipotassium ethylenediaminetetraacetic acid and lithium heparin on hematologic values in the green iguana (*Iguana iguana*). Journal of Zoo and Wildlife Medicine, 35: 328-332. doi: 10.1638/03-057.
- Korcock, D.E., Houston, A.H. and Gray, J.D. 1988. Effects of sampling conditions on selected blood variables of rainbow trout, *Salmo gairdneri* Richardson. Journal of Fish Biology, 33: 319-330. doi: 10.1111/j.1095-8649.1988.tb05474.x.
- Mariańska, B., Fabijańska-Mitek, J. and Windyga, J. 2003. Laboratory hematological analyses. Manual for medicine students. Wyd. Lek. PZWL, Warszawa.
- McDonough, C.J., Roumillat, W.A. and Wenner, C.A. 2005. Sexual differentiation and gonad development in striped mullet (*Mugil cephalus* L.) from South Carolina estuaries. Fishery Bulletin, 103: 601-619. <http://hdl.handle.net/10827/10565>.
- Morris, J.D., Fernandez, J.M., Chapa, A.M., Gentry, L.R., Thorn, K.E. and Weick, T.M. 2002. Effects of sample handling, processing, storage, and hemolysis on measurements of key energy metabolites in ovine blood. Small Ruminant Research, 43: 157-166. doi: S0921-4488(01)00266-8.
- National Committee for Clinical Laboratory Standards. 1996. Evacuated tubes and additives for blood specimen collection, 4th ed.; approved standard. NCCLS Document H1-A4. Villanova, PA: NCCLS.
- Olsen, A.K., Bladbjerg, E.M., Jansen, A.L. and Hansen, A.K. 2001. Effect of pre-analytical handling on haematological variables in minipigs. Laboratory Animals, 35: 147-152. doi: 10.1258/0023677011911516.
- Smit, G.L., Hattingh, J. and Schoonbee, H.B. 1977. Observations on some effects of disodiummethylen ediamine tetra-acetate and heparin on fish blood. Comparative Biochemistry and Physiology, 57: 35-38. doi: 10.1016/0306-4492(77)90074-0.
- Smith, C. 2007. Hematology and Cell Type Differentiation. In: S. Mumford, J. Heidel, C. Smith, J. Morrison, B. MacConnell and V. Blazer (Eds.), Fish Histology and Histopathology, USFWS-NCTC, 4: 1-12.
- Svobodova, Z., Pravda, D. and Palackova, J. 1991. Unified methods of haematological examination of fish. Research Institute of Fish Culture and Hydrobiology, Methods Notes, Vodnany: 20-31.
- Walencik, J. and Witeska, M. 2007. The effects of anticoagulants on hematological indices and blood cell morphology of common carp (*Cyprinus carpio* L.). Comparative Biochemistry and Physiology, 146: 331-335. doi: 10.1016/j.cbpc.2007.04.004.
- Witeska, M. and Wargocka, W. 2011. Disodium EDTA used as anticoagulant causes hemolysis in common carp blood. Turkish Journal of Veterinary and Animal Sciences, 35: 99-104. doi:10.3906/vet-0908-51.
- Wood, B.L., Andrews, J., Miller, S. and Sabbath, D.E. 1999. Refrigerated storage improves the stability of complete blood count and automated differential. American Journal of Clinical Pathology, 112: 687-695. PMID: 10549256.