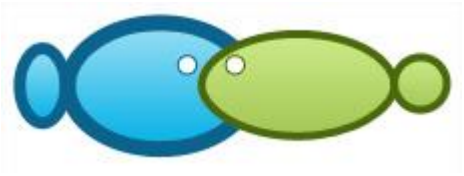

Prespawning and postspawning hematological parameters and oxidative stress biomarkers of brown trout (*Salmo trutta*) (Pisces: Salmonidae) from Someșul Cald River

[1]Lațiu Călin, [1]Cocan Daniel, [1]Uiuiu Paul, [2]Matei Maria-Cătălina, [1]Constantinescu Radu, [1]Hegheduș Cristina, [1]Ihuț Andrada, [1]Răducu Camelia, [3]Alexandru S. Nicula, [1]Mireșan Vioara

[1] Faculty of Animal Science and Biotechnologies, University of Agricultural Sciences and Veterinary Medicine Cluj-Napoca, Romania; [2] Faculty of Veterinary Medicine, University of Agricultural Sciences and Veterinary Medicine Cluj-Napoca, Romania; [3] National Institute for Economic Research "Costin C. Kiriţescu", Romanian Academy, Bucharest, Romania. Corresponding author: V. Mireșan, vioara.miresan@usamvcluj.ro

Abstract. Brown trout is one of the most studied fish species from the Salmonidae family, along with rainbow trout *Oncorhynchus mykiss* and brook trout *Salvelinus fontinalis,* because of its economic importance, game fish qualities, and adaptation to new environments. The aim of the present study was to assess the impact of the spawning period on hematological parameters and oxidative stress biomarkers for resident brown trout *Salmo trutta*. A total number of 59 specimens were caught using single-pass electrofishing techniques (n=32 for the prespwaning period; n=27 for the postspawning period) from Someșul Cald River in September and November 2018. Under anesthesia, blood samples were collected in Li-Heparin tubes and stored at 4^{o}C. Hematological parameters, oxidative stress biomarkers, and water samples were assessed and the results were statistically interpreted. The obtained results showed variations between prespawning and postspawning periods. The red blood cells count (RBC) was 1.79-3.14 mil mm^{-3} prespawning and 2.6-5.07 mil mm^{-3} postspawning. The superoxide dismutase (SOD) was 800.9-1140 U gHb^{-1} prespawning and 710-998.6 U gHb^{-1} postspawning. The highest coefficient of variation was recorded for mean corpuscular hemoglobin concentration (MCHC) postspawning (21.78%) while the lowest percentage was recorded for hematocrit (Hct) prespawning (6.03%). The unpaired t-test showed that the differences were statistically significant ($p<0.05$).
Key Words: electrofishing, GPx, hematology, river resident, SOD.

Introduction. The spawning period of brown trout *Salmo trutta* (Linnaeus, 1758), in general, is from September to December, rarely in January, when the water temperature is between 6 and 8^{o}C (Bănărescu 1964; Kottelat & Freyhof 2007). Fish may injure themselves (mechanical injuries) or can be injured by other fish during spawning activities (Greeley 1932; Allison 1951). This can change the levels of reactive oxygen species (ROS) in tissue like skin, muscle, fins and membranes (Ardiansyah et al 2020). Superoxide dismutase (SOD) and glutathione peroxidase (GPx) dosages may activate intrinsic or extrinsic pathophysiological mechanisms that can lead to increased oxidative stress responses (Wagner et al 2017).

The relationship between free radicals and antioxidants is generally characterized by inverse correlation. Oxidative stress is a result of the cellular response mechanism to the imbalance between oxidants and antioxidants. Environmental conditions, food availability, and spawning season can break the balance between free radicals and antioxidants. High levels of free radicals can cause the oxidation of proteins and lipids and, most importantly, DNA oxidation.

Oxidative stress has a great impact on environmental and aquatic toxicology. Moreover, the knowledge in this field can be easily used to determine polluted waters, with the purpose to enrich the wellbeing of fish (Slaninova et al 2009).

Environmental conditions have a strong impact on oxidative stress markers for fish. For example, changes in water temperature can produce thermal stress. In addition, water pollution and ultraviolet radiations are other factors with strong impact. All these changes can lead to the production of ROS and, to oxidative stress in organisms that cannot detoxify ROS, consequently leading to the inability to repair injuries (Halliwell & Gutteridge 1999; Ahmed 2005; Madeira et al 2013). In aquatic environments, where all these factors are easily disturbed, the production of ROS by organisms is very common (Lesser 2006; Madeira et al 2013). On the other hand, all those active species are synthesized in a natural way by cells, playing an important role in cellular activity, mostly being implicated in intracellular signaling. However, if the organisms are exposed to environmental stress for a long period, the capacity to adapt to the stressor is surpassed, so ROS can produce injuries to a cellular level (Madeira et al 2013).

Organisms developed over time some mechanisms to adapt to ROS that produces injuries. Therefore, cells are equipped with antioxidant defenses such as antioxidant enzymes (AOX) and non-enzymatic compounds like amino acids and vitamins (Madeira et al 2013). In all organisms, the antioxidant enzymes most important for detoxification of ROS are SOD, catalase (CAT), GPXs, transferases, xanthine oxidase, and glucose 6-phosphate dehydrogenase (Di Giulio & Meyer 2008).

Red blood cells parameters analysis is considered to be a minimally invasive procedure that is able to provide information about the physiological condition of fish. However, blood values can register a wide variation among different species and, as a response to different environmental conditions or physiological states, including the spawning period (Campbell 2012). A recurrent problem is that reference intervals for wild populations are lacking for most species, or are available for captive individuals (laboratory or fish farms) (Knowles et al 2006). The aim of the study was to provide basic information and reference on SOD and GPx values during pre and post spawning period of brown trout, in the context of fish welfare practices (wild brown trout).).

Material and Method. Brown trout specimens and blood samples were harvested on September 1st and November 28th, 2018 from Someșul Cald River, Cluj County, Romania. Electrofishing techniques were used for *S. trutta* sampling (Bohlin et al 1989). Blood was collected from the caudal vein and transferred in 4 mL Lithium-Heparin tubes (Sihoka & Wagenaar 2018). Hemoglobin (Hb), hematocrit (Hct), red blood cell count (RBC), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), SOD, and GPx were determined at the UASVM Cluj-Napoca hematology laboratory. Statistical analyses were performed with GraphPad and MS Excel.

Sampling site. Someșul Cald River together with Someșul Rece River form Someșul Mic River with a length of 178 km. They are part of the Someș-Tisa river basin. Its surface is 3773 km^2. Someșul Cald has a total length of 66.5 km and an area of 331 km^2. It springs from the Bihariei-Vlădeasa massif below the Piatra Arsă and Cârligatele peaks (Duma 2016) (altitude of 1550 m) and is the largest river that supplies the Beliș accumulation lake (Figure 1).

Fish sampling and water parameters. *S. trutta* specimens were sampled using single-pass electrofishing techniques (Ainslie et al 1998), using pulsed DC on a Samus 725 MP electrofishing unit apparatus, powered by 12V and 24 A rechargeable battery. Physico-chemical water parameters are fundamental regulatory factors for the lives of fish (Pankhurst & Munday 2011) and were tested before electrofishing: pH, temperature, conductivity, total dissolved solids (TDS), salinity, transparency, hardness, NO_3, NO_2, NH_3 using a Hanna HI9828 water multi-parameter.

Fish anesthesia and blood sampling. Prior to blood sampling, fish were anesthetized using Eugenol (4-Allyl-2-methoxyphenol), a natural essential oil extracted from cloves. Clove oil induces loss of mobility, loss of sensations, equilibrium, and loss of reflex to fish (Anderson et al 1997; Cocan et al 2019), and it was also used for stress indicator analysis (Wynn & Fougere 2006). The used dosage was 30 mg L^{-1} according to Palić et al (2006). Blood samples were collected when fish were in dorsal decubitus during the anesthesia procedure. After blood sampling, fish were placed in a well-oxygenated water tank until full recovery from the procedure***.***

The widespread use of hematology for assessing the health of fish is a common and simple procedure (Pickering 1986). From each anesthetized individual, ≈4 mL of blood was collected from the caudal vein and transferred into Lithium-Heparin blood sample tubes (Coroian et al 2019).

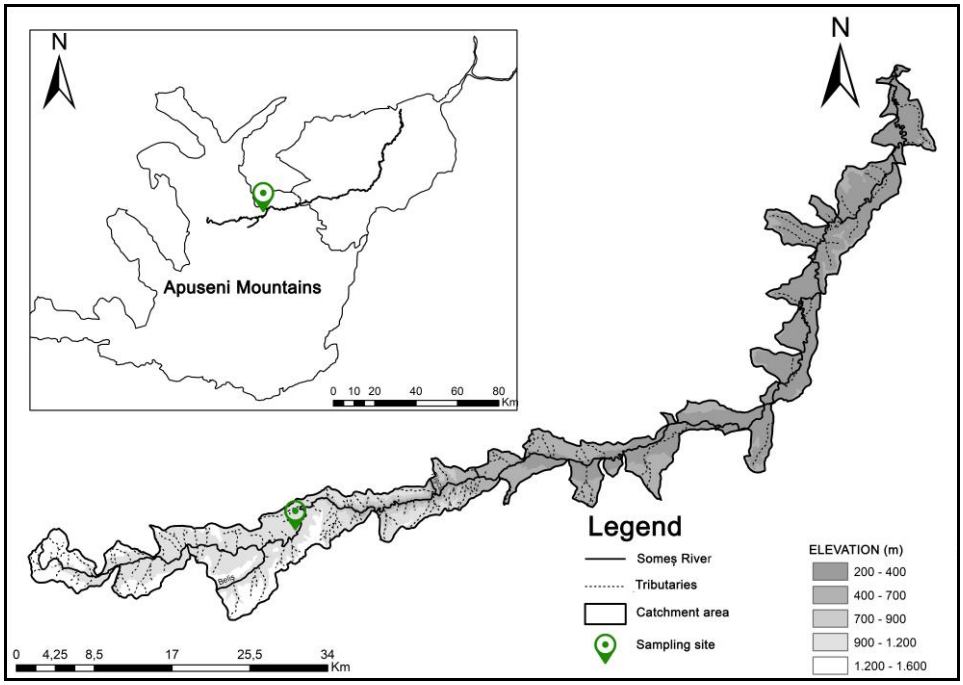

Figure 1. Someşul Cald River.

Hematological analysis. Hematological assessments included RBC, packed cellular volume (PCV) or Hct, Hb, MCV, MCH, and MCHC parameters. The samples used were represented by whole blood sampled on Li-heparin. Erythrocytes were determined by a visible turbidimetric reaction (λ=546 nm) at 37°C, using Gowers reactive. Hemoglobin dosage was carried out in a maximum of 6 hours after sampling, using an END-POINT type colorimetric reaction, read in the VIS spectrum at λ=546nm, 37°C. PCV assessment was carried out by centrifugation in capillary tubes at 12000 rotations min^{-1}, for 3 min. Erythrocyte indices were calculated following the indications of Ognean & Cernea (2006).

Parameters of oxidative stress assessment - SOD and GPx. Using the methods of Prohaska & Ganther (1977), Kraus & Ganther (1980), Arthur & Boyne (1985), and Suttle (1986), , the antioxidant enzymes SOD and GPx were assessed.

For SOD, the principle of the reaction was based on the role of SOD to accelerate the superoxide radical (O_2)- dismutation process into hydrogen peroxide and molecular oxygen. The whole blood was centrifugated 10 min at 3000 rotations per minute in order to separate the blood plasma. After separation, plasma was aspirated and the remaining cellular mass was washed 4 more times with 3 mL of NaCl 0.9%. The remaining liquid was eliminated by aspiration. The prepared erythrocytes were lysed with 2 mL of cool distilled water, mixed and left at 2-4°C for 15 min. The lysate was diluted with phosphate buffer (pH=7) in order to lower the inhibition rate from 60% to 30%. The next step

consisted in seriated dilutions for the standard in order to obtain the calibration curve. The sample was mixed with reactive 1 (from the used kit) and kept at 37°C for 1 min. After xanthine oxidase was added, the sample was read at λ=505nm, 37°C. Using the calibration curve, the inhibition rate was calculated. The final result was obtained by the transformation in USOD gHb^{-1}.

For GPx determination, the sample was represented by whole blood on Li-heparin. The dilution of the sample was carried out with 3mL of dilution buffer. The next steps were done according to the kit instructions: blank dosage and diluted sample dosage. The final result was obtained by subtracting the blank value from each sample, obtaining the GPx value from the blood sample, measured in enzymatic units g^{-1} Hb (UGPx gHb^{-1}).

Data analysis. Data was processed in MS Excel and descriptive statistics (mean values, minimum and maximum values, standard deviation, and standard error of the mean) and unpaired t-test (p-value, 95% confidence interval, R^2) were performed using GraphPad Prism 8 software.

Results and Discussion

Water parameters. The coefficient of variation (CV%) for temperature indicated significant changes, water temperature decreasing with 6.3°C (from 11.4°C to 5.1°C). The values of pH were 6.8 during prespawning and 7.2 during postspawning, indicating an almost neutral aquatic environment. The conductivity of water was influenced by the amount of ions dissolved, in this case, the variation of the two periods being low (Table 1). TDS values were highly similar (0.04–0.05). Salinity was identical in both sampling periods. Transparency was higher in the postspawning period due to low precipitations in the area. Hardness was higher during prespawning. Regarding nitrates, a higher value was recorded during the prespawning period, while nitrites were not present in the sampled water. Ammonia levels were highly similar in both periods (0.0003–0.0002 mg L^{-1}).

Table 1

Pre and postspawning water parameters from Someșul Cald River at the time of *Salmo trutta* blood sampling (prespawning in September; postspawning in November)

Period	*Temp °C*	*pH*	*Cond µS cm^{-1}*	*TDS mg L^{-1}*	*Sal g L^{-1}*	*Transp cm*	*Hardness °dGH*	*NO_3 mg L^{-1}*	*NO_2 mg L^{-1}*	*NH_3 mg L^{-1}*
PRE	11.4	6.8	85.14	0.04	0.04	108.27	3.7	13.86	0	0.0003
POST	5.1	7.2	68.2	0.05	0.04	120.2	2.98	9.21	0	0.0002
Mean	8.25	7	76.67	0.045	0.04	114.2	3.34	11.54	0	0.00025
SD	4.46	0.28	11.98	0.007	0	8.44	0.51	3.29	0	0.00007
SEM	3.15	0.2	8.47	0.005	0	5.97	0.36	2.33	0	0.00005
Diff.	6.3	-0.4	16.94	-0.01	0	-11.93	0.72	4.65	0	0.0001
CV%	54	4.04	15.62	15.71	0.00%	7.38%	15.24%	28.5%	0%	28.28%

Note: PRE - prespawning; POST - postspawning; Mean - mean values; SD - standard deviation; SEM - standard error of mean; Diff - differences (Prespawning - Postspawning); CV% - coefficient of variation; Temp - Temperature; Cond - conductivity; TDS - total dissolved solids; Sal - salinity; Transp - transparency; NO_3 - nitrate; NO_2 - nitrite; NH^3 - ammonia.

Hematology. For the hematological parameters, the descriptive statistics analysis shows a variance between the values recorded, both for the prespawning and postspawning moments of sampling. Mean values recorded for RBC and Hb showed increased values during the postspawning period. MCV mean values are higher during the prespawning period, while Hct, MCH, and MCHC show highly similar values (Tables 2 and 3). The t-test (unpaired) showed significant differences for all the hematological parameters ($p<0.05$) (Figure 2).

Oxidative stress biomarkers (SOD and GPx). The same trend of evolution was recorded for the oxidative stress determined parameters. For both oxidative stress biomarkers (SOD and GPx), the mean values are higher in the prespawning period (Table 4). The t-test (unpaired) underlined that the differences are statistically significant ($p<0.05$) (Figure 3).

Table 2

Hematological parameters of *Salmo trutta* from Someșul Clad River

Hematological parameters		*RBC (mil mm^{-3})*		*Hct (%)*		*Hb (g dL^{-1})*	
		Prespawning (n=32)	*Postspawning (n=27)*	*Prespawning (n=32)*	*Postspawning (n=27)*	*Prespawning (n=32)*	*Postspawning (n=27)*
Descriptive statistics	Mean	2.58	3.546	42.56	47.07	9.953	12.46
	Min-Max	1.79-3.14	2.6-5.07	38-47	35-58	8-11.9	9.8-16.9
	SD	0.4141	0.5597	2.564	5.622	1.119	2.182
	SEM	0.07321	0.1077	0.4533	1.082	0.1979	0.4199
	CV%	16.05%	15.78%	6.03%	11.94%	11.25%	17.52%
t test (unpaired)	p value	<0.0001		0.0001		< 0.0001	
	P<0.05	yes (****)		yes (***)		yes (****)	
	CI 95%	0.7117 to 1.22		2.292 to 6.731		1.619 to 3.386	
	R square	0.5038		0.2252		0.3606	

Note: RBC - red blood cells; Hct - hematocrit; Hb - hemoglobin; n - number of samples; Min and Max - minimum and maximum recorded values, respectively; SD - standard deviation; SEM - standard error of mean; CV% - coefficient of variation; CI - confidence interval; R square - coefficient of determination.

Table 3

Erytrocitary indices of *Salmo trutta* from Someșul Clad River

Erytrocitary indices		*MCH (pg)*		*MCV (fL)*		*MCHC (g dL^{-1})*	
		Prespawning (n=32)	*Postspawning (n=27)*	*Prespawning (n=32)*	*Postspawning (n=27)*	*Prespawning (n=32)*	*Postspawning (n=27)*
Descriptive statistics	Mean	39.28	35.59	168.6	134.7	2.342	2.687
	Min-Max	22.97-54.75	23.29-44.3	129-223.5	108.8-196.2	1.91-2.95	1.9-3.98
	SD	6.238	6.166	24.91	20.72	0.2678	0.5852
	SEM	1.103	1.187	4.403	3.987	0.04734	0.1126
	CV%	15.88%	17.33%	14.77%	15.38%	11.43%	21.78%
t test (unpaired)	p value	0.0266		<0.0001		0.0042	
	P<0.05	yes (*)		yes (****)		yes (**)	
	CI 95%	-6.939 to -0.4446		-45.94 to -21.77		0.1133 to 0.5757	
	R square	0.08335		0.3558		0.1351	

Note: MCH - mean corpuscular hemoglobin; MCV - mean corpuscular volume; MCHC - mean corpuscular hemoglobin concentration; n - number of samples; Min and Max - minimum and maximum recorded values, respectively; SD - standard deviation; SEM - standard error of mean; CV% - coefficient of variation; CI - confidence interval; R square - coefficient of determination.

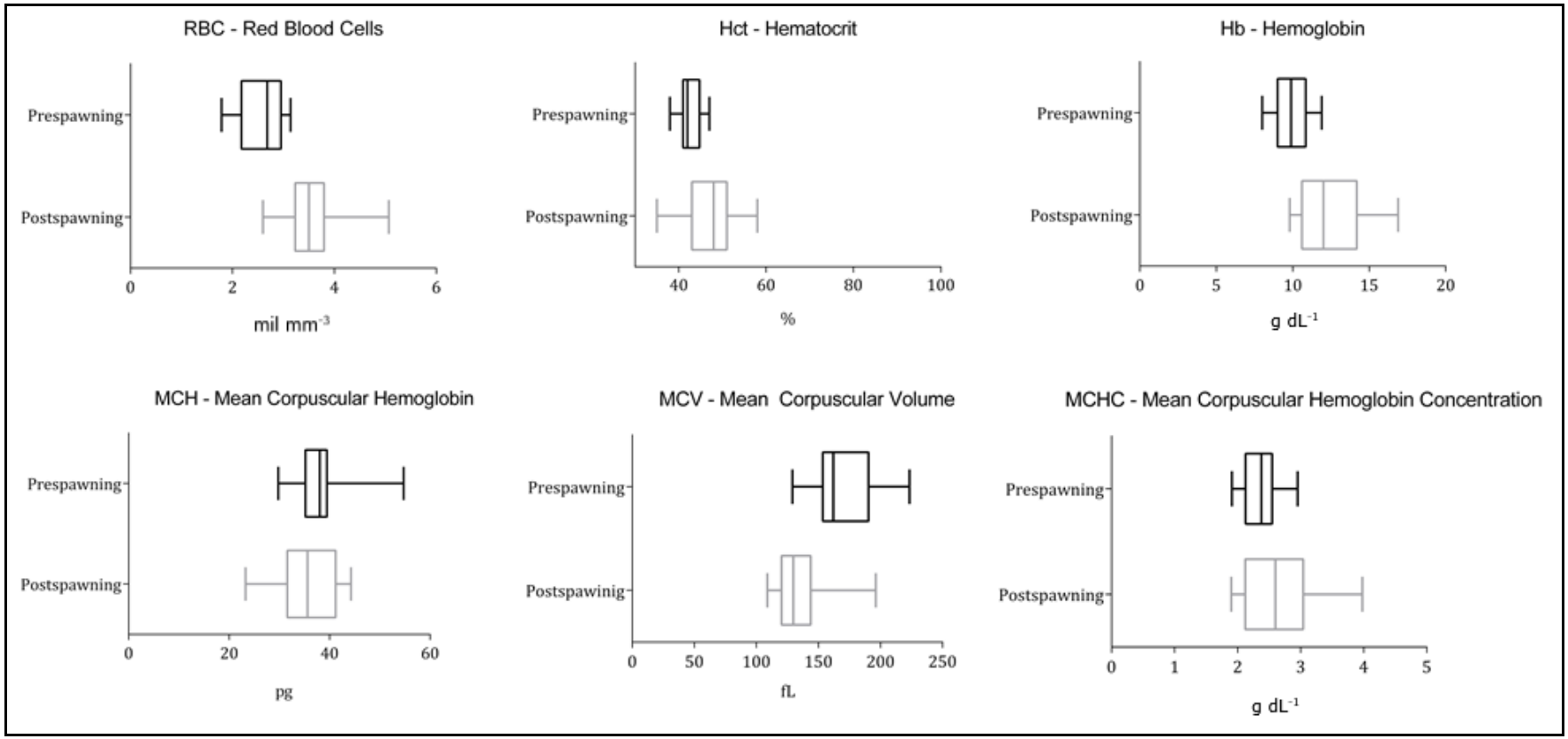

Figure 2. Comparative hematological parameters of *Salmo trutta*, pre and postspawning sampling.

Table 4

Oxidative stress biomarkers superoxide dismutase and glutathione peroxidase

Antioxidant enzymes		*SOD (U gHb^{-1})*		*GPx (U gHb^{-1})*	
		Prespawning (n=32)	*Postspawning (n=27)*	*Prespawning (n=32)*	*Postspawning (n=27)*
Descriptive statistics	Mean	921.9	837.6	112	94.86
	Min-Max	800.9-1104	710-998.6	92.4-133.2	78-119.2
	SD	65.91	71.11	11.08	10.45
	SEM	11.65	13.69	1.958	2.011
	CV%	7.15%	8.49%	9.89%	11.02%
t test (unpaired)	p value	<0.0001		<0.0001	
	P<0.05	yes (****)		yes (****)	
	95% confidence interval	-120.1 to -48.6		-22.77 to -11.47	
	R square	0.2814		0.3924	

Note: SOD - superoxide dismutase; GPx - glutathione peroxidase; n - number of samples; Min and Max - minimum and maximum recorded values, respectively; SD - standard deviation; SEM - standard error of mean; CV% - coefficient of variation; R square - coefficient of determination.

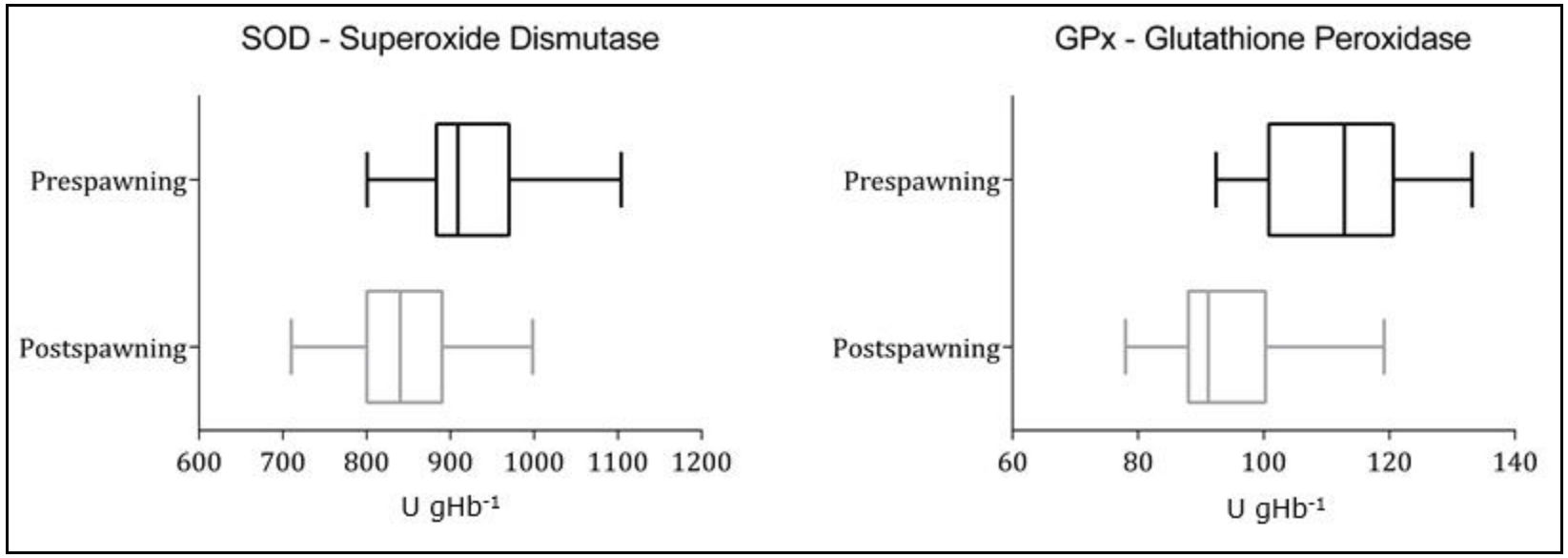

Figure 3. Comparative oxidative stress enzymes of *Salmo trutta* from Someșul Cald River, pre and postspawning sampling.

The spawning period produces a series of modifications in the body of fish. However, these variances are considered physiological, as long as reproductive processes (spermiation, ovulation, embryogenesis, and hatching) are normal in any animal organism (Pankhurst & Munday 2011). Hematological parameters are frequently used as a marker for the general health status of the organisms (Chowdhury et al 2020). The advantages of blood parameter assessments are numerous. Blood sampling is a minimally invasive procedure, the costs of the determinations are not high and, more importantly, a complete blood panel is able to provide enough information regarding the physiological state of the body (Campbell 2012). Moreover, in wild fish populations, the options are limited when it comes to the evaluation of the clinical status, so assessing blood parameters remains a good option in order to gain more information regarding the population's life and clinical status.

In the present study, the impact of the spawning period on blood parameters, more exactly on red blood cell parameters and on two enzymes dosed to evaluate oxidative stress (SOD and GPx) was identified. After the comparison between values recorded for the RBC indices on the prespawning and postspawning periods, a variance could be observed. However, since there are no available reference intervals for *S. trutta* blood parameters (Campbell 2012), especially in the spawning period, the interpretation of the results should be made taking into account all data available. Thus, even if a variance was noted, it can be considered a physiological one or, with some reserves, it can be attributed to the spawning period and it cannot be considered to have a pathological significance.

SOD and GPx are antioxidant enzymes with a great role in the body's ability to neutralize ROS (Di Giulio & Meyer 2008). Because the activity of these enzymes is variable between species and also between individuals from the same species, the possibility to establish some reference intervals is almost impossible. So, as in the case of red blood cell parameters, the values of SOD and GPx values should be interpreted taking into account all the data available. For the individuals included in the present study, the activity of those two antioxidant enzymes can be observed. The variance in values between the time of the dosage and between the individuals was observed. This data variability cannot be attributed to the spawning period, but it can be considered a normal response of the fish's organism to the presence of ROS. Previous studies analyzed the oxidative stress in fish in a different context. Madeira et al (2013) analyzed the activity of antioxidant enzymes in estuarine fish under different temperature influence. Their results showed that not all species had the same reaction to the increase of the temperature when it came to antioxidant enzymes. This can be explained by the fact that the enzymes have a different activity between species and between individuals. However, their general conclusion supported the previous knowledge, that the change of temperature has an influence on the parameters of oxidative stress in poikilotherm organisms (Madeira et al 2013). In the current study, water temperature registered a normal variation between the prespawning and postspawning period. This change is normal, due to the different time of the year when it was registered (September vs. November). It cannot be concluded that this variation had a direct or significant impact on the antioxidant enzyme values, while the values did not have a specific pattern in evolution.

Slaninova et al (2009) remarks the same detail, that the manifestation of oxidative stress is variable in terms of fish species and organs, in the context of polluted waters. In the current study, the water analysis results do not show any indication of pollution, so the variation of oxidative stress markers cannot be attributed to the water quality.

Conclusions. The results of this study provide new information regarding hematological parameters and oxidative stress biomarkers in wild *Salmo trutta* from Someșul Cald River. Moreover, the findings may be used in the context of brown trout breeding, both for artificial reproduction and natural spawning. Future studies are required in order to establish if the spawning period (not only the time period but also the whole physiological mechanisms) influences the oxidative stress biomarkers: antioxidant enzymes system,

catalase (CAT), malonildealdehide (MDA), total antioxidant capacity (T-AOC), etc., and also non-enzymatic systems (vitamins). Moreover, a complete blood panel should be assessed (complete red blood cell parameters, complete white blood cell count, and leucocitary formula).

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Authors:
Călin Laţiu, Faculty of Animal Science and Biotechnologies, University of Agricultural Sciences and Veterinary Medicine Cluj-Napoca, 3-5 Mănăştur St., 400372 Cluj-Napoca, Romania, e-mail: calin.latiu@usamvcluj.ro
Daniel Cocan, Faculty of Animal Science and Biotechnologies, University of Agricultural Sciences and Veterinary Medicine Cluj-Napoca, 3-5 Mănăştur St., 400372 Cluj-Napoca, Romania, e-mail: daniel.cocan@usamvcluj.ro
Paul Uiuiu, Faculty of Animal Science and Biotechnologies, University of Agricultural Sciences and Veterinary Medicine Cluj-Napoca, 3-5 Mănăştur St., 400372 Cluj-Napoca, Romania, e-mail: paul.uiuiu@usamvcluj.ro
Maria-Cătălina Matei, Faculty of Veterinary Medicine, University of Agricultural Sciences and Veterinary Medicine Cluj-Napoca, 3-5 Mănăştur St., 400372 Cluj-Napoca, Cluj, Romania, e-mail: catalina.matei@usamvcluj.ro
Radu Constantinescu, Faculty of Animal Science and Biotechnologies, University of Agricultural Sciences and Veterinary Medicine Cluj-Napoca, 3-5 Mănăştur St., 400372 Cluj-Napoca, Romania, e-mail: radu.constantinescu@usamvcluj.ro
Cristina Hegheduș, Faculty of Animal Science and Biotechnologies, University of Agricultural Sciences and Veterinary Medicine Cluj-Napoca, 3-5 Mănăştur St., 400372 Cluj-Napoca, Romania, e-mail: cristina.hegedus@usamvcluj.ro
Andrada Ihuț, Faculty of Animal Science and Biotechnologies, University of Agricultural Sciences and Veterinary Medicine Cluj-Napoca, 3-5 Mănăştur St., 400372 Cluj-Napoca, Romania, e-mail: andrada.ihut@usamvlcuj.ro
Camelia Răducu, Faculty of Animal Science and Biotechnologies, University of Agricultural Sciences and Veterinary Medicine Cluj-Napoca, 3-5 Mănăştur St., 400372 Cluj-Napoca, Romania, e-mail: camelia.raducu@usamvcluj.ro
Alexandru Sabin Nicula, National Institute for Economic Research "Costin C. Kiriţescu", Romanian Academy, Casa Academiei Române, 13 Septembrie St., RO-05071 Bucharest, Romania, e-mail: sabin.nicula @gmail.com
Vioara Mireșan, Faculty of Animal Science and Biotechnologies, University of Agricultural Sciences and Veterinary Medicine Cluj-Napoca, 3-5 Mănăştur St., 400372 Cluj-Napoca, Romania, e-mail: vioara.miresan@usamvcluj.ro

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