The Effect of Saline Solution Used as an Activating Factor of the Semen in Brown Trout, *Salmo Trutta Fario* (Linnaeus 1758)

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ABSTRACT

In this research we analysed the phenotypic characterization of *Salmo trutta fario* species and we studied the effect of the saline solution used as an activator for seminal material. The spermatozoa mobility ratio in our study ranged from 60% to 95%, with an average of 74% when using water and 84.5% when a saline solution (NaCl) of 7‰ was used. The average mobility time ranges between 58.7 seconds (water) and 99 seconds (saline solution). Biochemical analyses of seminal plasma were performed and parameters such as ions concentration (Na^+, K^+, Ca^{2+}, Mg^{2+}, Cl^-), protein profile (BUN, TP), lipid profile (TG, Chol) and enzyme profile (ALP) were followed. As a result of our research on semen in brown trout, we noticed that saline solution (7‰) compared to water when used as an activator of seminal material, is more effective in activating spermatozoa.

Keywords: *mobility duration, seminal plasma, spermatocrit, viability*

INTRODUCTION

The brown trout, *Salmo trutta fario* (Linnaeus, 1758) is a species of great importance in fish farming, so an assessment of sperm quality can contribute to an increase in the effectiveness of artificial fertilization (Dziewulska and Domagala, 2002).

In Salmonids, the disposal of semen is done externally, a period of time being necessary between sperm release and fertilization of eggs during which sperm have to retain fertilization capacity (Lahnsteiner *et al*., 1998, Cosson, 2004). Thus, to gather high rates of fertilization, it is necessary to achieve several factors, such as mobility (Alavi and Cosson, 2005, 2006; Curon *et al*., 1999; Rurangwa et al., 2004) viability (Hajirezaee *et al*., 2010; Ingermann *et al*., 2002; Lahnsteiner, 2007; Nynca and Ciereszko, 2009), spermatocrit and sperm density (Ciereszko *et al*., 2010; Lahnsteiner *et al*., 2010), factors that are interdependent.

Salmonids spermatozoa, as well as in other species, are immobile in the seminal tract and are activated only in the dilution with a suitable fertilization medium (Billiard, 1987a; Ingermann et al., 2008). Other studies were conducted to determinate a proper solution for the activation of semen (Nynca *et al*, 2012; Woolsey *et al*, 2006; Zuccarelli *et al*., 2007), thus, saline media of different concentrations were used (Billard, 1978; Billard, 1987b; Brown *et al*., 1994).

MATERIALS AND METHODS

The biological material used in this study was sampled from Fiad trout farm, Bistriţa-Năsăud County. The number of specimens from which samples were taken was 10 males, 6 years of age. Clinically healthy specimens were used in order

Tab. 1. The chemical composition of administered feed (Source:www.aller-aqua.com)

Specification	Granulation	
	6 mm	8mm
Crude protein (%)	53	53
Crude fat (%)	14	14
NFE (%)	15,5	15,5
Ash (%)	8,2	8,2
Fiber (%)	1,3	1,3
Digestible energy (MJ)	19,2	19,2
Vitamin A (IU)	10.000	10.000
Vitamin D_3 (IU)	1.000	1.000
Vitamin E (IU)	200	200

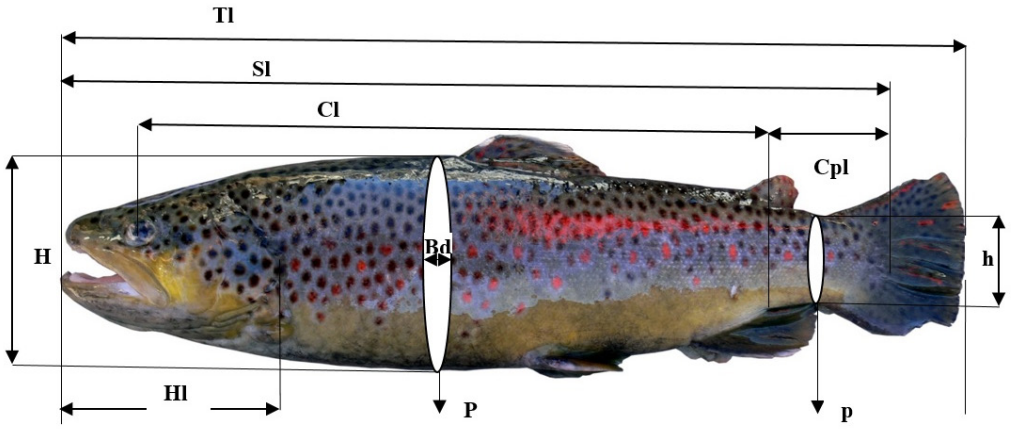

Fig. 1. Somatic measurements made in brown trout males
Note: **Tl** – total length; **Sl** – standard length; **Cl** – commercial length; **H** – maximum height; **h** – minium height; **Hl** – head length; **Cpl** – caudal peduncle length; **P** – large perimeter; **p** – small perimeter; **Bd** – body depth.

not to negatively affect the obtained results. The study took place during December 2015-January 2016. The feed administrated to the breeders group (Tab. 1) was Aller Aqua, Aller Rep Ex 6 - 8 mm.

To obtain the semen samples, the trout specimens were anesthetized prior to harvesting with clove oil, 0.047 ml / L concentration in water (*Eugenia caryophyllata*), a solution used as a local antiseptic and anesthetic. After anesthetization, with a gentle pushing motion along the abdomen, the semen was transferred into Eppendorf tubes. Immediately after harvesting of the semen, the Eppendorf tubes were introduced in an isothermal bag at a temperature of 4 °C.

For the phenotypic characterization of the species, 11 somatic measurements were performed (Fig.1), which were made on the digital photographs taken, with ToupView-AmScope software.

To determine sperm mobility and viability (Fig. 2), water and saline solution (NaCl - 7 ‰) were used. 1 µl of semen was diluted in 20 µl of water and 1 µl of semen was diluted in 20 µl of saline solution (NaCl - 7‰). The microscope used was equipped with a Nikon D300 camera and 60x objective was used.

The sperm density was performed by microscopic examination; sperm count was made in one millilitre of semen, using the *Bürker-Türk* chamber. Mett tubes were used to determine the spermatocrit, which was centrifuged at 4500 rpm for 5 minutes to separate seminal plasma from spermatocrit.

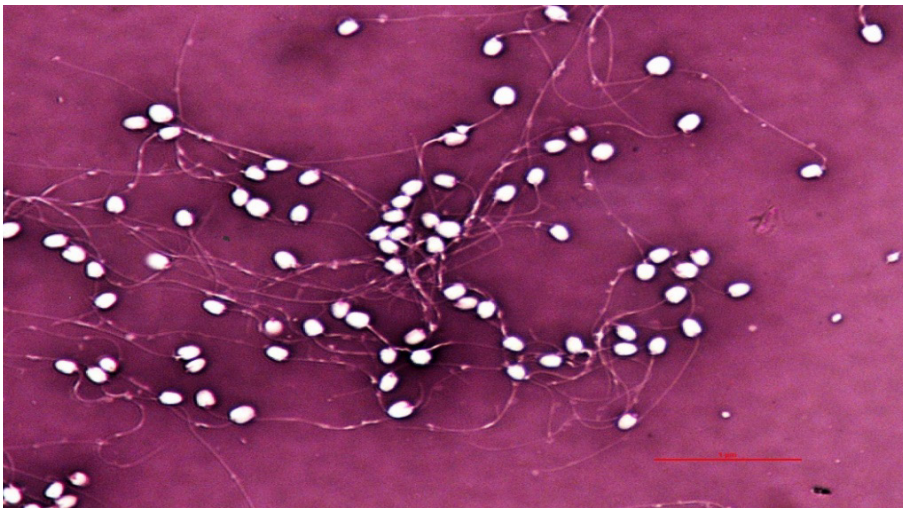

Fig. 2. Spermatozoa captured from microscope using Nikon D300 camera

Tab. 2. Wavelength reading (λ) for biochemical analyses on seminal plasma in brown trout

Specifications	Abbr.	MU	λ
Mineral profile			
Sodium	Na^+	mmol/L	405 nm
Potassium	K^+	mmol/L	380 nm
Calcium	Ca^{2+}	mg/dl	575 nm
Magnesium	Mg^{2+}	mg/dl	560 nm
Chlorine	Cl^-	mmol/L	520 nm
Protein profile			
Urea	BUN	mg/dl	340 nm
Total Protein	TP	g/dl	546 nm
Lipid profile			
Tryglicerides	TG	mg/dl	550 nm
Cholesterol	Chol	mg/dl	500 nm
Enzyme profile			
Alkaline phosphatase	ALP	U/L	405 nm

Biochemical analyses performed on seminal plasma were represented by the mineral profile: sodium (Na^+), potassium (K^+), calcium (Ca^{2+}), magnesium (Mg^{2+}), chlorine (Cl^-); protein profile: urea (BUN), total protein (TP); lipid profile: triglycerides (TG), cholesterol (Chol) and enzymatic profile: alkaline phosphatase (ALP). All analyses were performed by MasterTouch spectrophotometer by reading the wavelengths (Tab.2.) at the pre-set values.

The obtained data was interpreted and processed statistically with the GraphPad Prism v6 software. Drafting, images, graphics and spreadsheets were edited in Microsoft Word v. 2013 and Adobe Photoshop CC 2017. All the methods used are up-to-date and the existing data has been obtained in the laboratories of USAMV Cluj-Napoca, Faculty of Animal Science and Biotechnology, discipline Physiology of Aquatic Organisms.

RESULTS AND DISCUSSIONS

The mean body mass (Table 3) was 0.96 ± 0.07 kg with a minimum of 0.67 kg and a maximum of 1.39 kg. The coefficient of variability of body mass had an average of 22.23%. This shows the

Tab. 3. The mean values of somatic measurements in brown trout (*Salmo trutta fario*) males at age of six years (n=10)

Specification	Abbreviation	MU	X ± SEM	Minimum	Maximum	V%	SD
Body mass	Bw	kg	0.96 ±0.07	0.67	1.39	22.23	0.21
Total length	Tl	cm	42.37±0.92	38.90	48.82	6.84	2.90
Standard length	Sl	cm	38.84±0.85	35.53	44.64	6.91	2.68
Commercial length	Cl	cm	29.13±0.71	26.26	33.71	7.70	2.24
Maximum height	H	cm	9.99±0.29	8.48	11.57	9.34	0.93
Minimum height	H	cm	4.03±0.08	3.54	4.48	6.18	0.25
Head length	Hl	cm	10.08±0.20	9.21	11.63	6.34	0.64
Caudal peduncle length	Cpl	cm	5.95±0.12	5.41	6.66	6.45	0.38
Large perimeter	P	cm	30.56±1.09	25.53	36.81	11.34	3.47
Small perimeter	P	cm	12.47±0.31	10.70	13.83	7.78	0.97
Body depth	Bd	cm	6.59±0.38	5.23	8.88	18.31	1.21

Tab. 4. The mean values of maintenance indices in brown trout (n=10)

Indices	Abbreviation	X ± SEM	Minimum	Maximum	V%	SD
Fulton	K	6.72±0.35	5.17	8.54	16.51	1.11
Profile	Ip	3.90±0.08	3.61	4.32	6.23	0.24
Thickness	Ig	16.92±0.73	13.83	19.88	13.75	2.33
Kiselev	IK	1.28±0.03	1.17	1.45	7.70	0.10
Carnality 1	Ic1	25.99±0.38	23.92	27.69	4.66	1.21
Carnality 2	Ic2	15.33±0.22	13.74	16.20	4.50	0.69

existence of a good homogeneity of the breeding group.

Analysing the total length, it has an mean value of 42.37 ± 0.92 cm, its variability being 6.84%, with a minimum length of 38.9 cm and the maximum of 48.82 cm. Regarding the variability coefficient of the studied characters, average variables are recorded for body weight and body depth, at the opposite pole, with low variables, the other 9 somatic studied characters. Overall, these low variability values indicate that this group of breeders is homogeneous, and that frequent sorting of breeding groups is performed within the Fiad trout farm.

Body indices (Tab. 4.) help us in selection of specimens for the breeding nucleus. Thus, the Fulton condition factor (K) shows the fish maintenance status, the mean value of this body index is 6.72 ± 0.35, which confirms that the fish maintenance status is good.

The profile index (Ip) highlights the body shape of the fish and allows individuals from a population to fit into a particular type of profile. Thus, in our case we have a low index value (3.90 ± 0.08), showing that fish have a pronounced spine convexity, which is correlated with a rich muscle mass in the trunk region.

The thickness index (Ig) expresses the depth of the body in its most developed region reported to the standard length of the fish. Its mean value is high (16.92 ± 0.73), so the fish will show better development of the lateral muscles.

The quality index, Kiselev (IK), gives us information on quality of fish, based on Kiselev relationship. The mean value of this index is small (1.28 ± 0.03), this value denoting a richer muscle mass due to the fact that the specimens have a longer circumference than the length.

The carnosity index (Ic1 - Ic2) is the ratio of the caudal head or peduncle in the standard length of the body. This index record a mean value of 25.99 ± 0.38 (Ic1) and 15.33 ± 0.22 (Ic2), thus, the fish have a high percentage of meat. The fact that carnosity index values fluctuate around 20%, indicates that the studied specimens have proper

Tab. 5. The mean values of biochemical analysis of seminal plasma in brown trout (*Salmo trutta fario*) males at age of six years (n=10)

Specifications	Abbr.	MU	X ± SEM	Min.	Max.	V%	SD
Mineral profile							
Sodium	Na^+	mmol/L	107±1.04	94.00	124.00	9.72	10.403
Potassium	K^+	mmol/L	12.8±0.17	10.50	15.00	13.01	1.665
Calcium	Ca^{2+}	mg/dl	4.06±0.16	2.70	6.20	28.33	1.150
Magnesium	Mg^{2+}	mg/dl	2±0.054	1.30	3.10	27.18	0.544
Chlorine	Cl^-	mmol/L	104.8±0.91	94.60	121.50	8.70	9.116
Protein profile							
Urea	BUN	mg/dl	12.3±0.15	10.40	14.60	11.95	1.470
Total Protein	TP	g/dl	0.25±0.01	0.17	0.32	20.91	0.052
Lipid profile							
Tryglicerides	TG	mg/dl	4.13±0.07	3.20	5.20	16.66	0.688
Cholesterol	Chol	mg/dl	3.14±0.07	2.20	4.20	22.02	0.692
Enzyme profile							
Alkaline phosphatase	ALP	U/L	36.2±0.59	30.00	48.00	16.25	5.884

body development, are healthy and have a high percentage of meat.

The ionic composition and seminal plasma metabolites (Tab. 5) have a significant influence on the mobility and viability in fish semen.

In our study, in brown trout semen, K^+ concentration is 12.8 ± 0.17 mmol/L, lower concentration than in *Oncorhynchus mykiss* (30.4 ± 4.5 mmol/L, Glogowski *et al*., 2000), *Salmo trutta abanticus* (38 mmol/L; Bozkurt, 2008), *Carassius gibelio* (26.20 mmol/L, Taati *et al*., 2010) and higher than in *Salmo trutta macrostigma* (8.18 ± 0.03 mmol/L, Bozkurt *et al*., 2011) and in *Tinca tinca* (0.6 mmol / L; Linhart *et al*., 2003b). In salmonids, sperm motility is controlled by the K^+ concentration, since it has been known since 1938 that millmolar level of extracellular K^+ ion concentration in the seminal tract is primarily responsible for keeping spermatozoa inactive (Schenk and Kahmann, 1938).

Na^+ concentration is 107 ± 1.04 mmol/L, lower concentration than in *Oncorhynchus mykiss* (122 ± 14.2 mmol/L; Glogowski *et al*., 2000), *Salmo trutta macrostigma* (121 ± 0.37 mmol/L; Bozkurt *et al*., 2011), but higher than in *Carassius gibelio* (101.59 mmol/L, Taati *et al*., 2010) and *Tinca tinca* (18.40 ± 1.3 mmol/L; Linhart *et al*., 2003b). Sperm mobility also depends on other ions (Ca^{2+}, Mg^{2+}), which presents a significant importance in initiating sperm mobility (Linhart *et al*., 2003a).

The mean values in Ca^{2+} and Mg^{2+} are 4.06 ± 0.16 mg/dl respectively 2 ± 0.05 mg/dl, , values higher than in *Oncorhynchus mykiss* (Ca^{2+}= 1.10 ± 0.26 mg/dl; Mg^{2+}= 0.85 ± 0.12 mg/dl; Gallowski *et al*., 2000), *Carassius gibelio* (Ca^{2+}= 101.59 mg/dl, Mg^{2+}= 0.85 ± 0.12 mg/dl, Taati *et al*., 2010), but lower than in *Salmo trutta macrostigma* (Ca^{2+}= 7.23 ± 0.03 mg/dl; Mg^{2+}= 3.19 ± 0.02 mg/dl; Bozkurt *et al*., 2011). The bivalent cations (Ca^{2+}, Mg^{2+}) are much more effective in suppressing the K^+ inhibitory effect on sperm motility than Na^+. The effect of inhibiting K^+ mobility can be counteracted by increasing the concentration of Ca^{2+} from outside (Billiard and Cosson, 1992). The total protein from the seminal plasma has a mean value of 0.25 ± 0.01 g/dl, a value similar to that of *Salmo trutta macrostigma* (0.48 ± 0.02 g/dl, Bozkurt *et al*., 2011). Total protein has an important protective role for spermatozoa and contains a large number of key enzymes in the metabolic process. In terms of urea (BUN), higher concentrations were recorded, 12.3 ± 0.15 g/dl, compared to *Salmo trutta macrostigma* (9.97 ± 0.39 g/dl, Bozkurt *et al*., 2011). Contamination of sperm with urea can reduce sperm motility and fertilization capacity (Nynca *et al*., 2012).

Regarding the lipid profile, the mean triglyceride value is 4.13 ± 0.07 mg/dl and cholesterol of 3.14 ± 0.07 mg/dl. Triglycerides act as sperm energy sources, both in the immobility

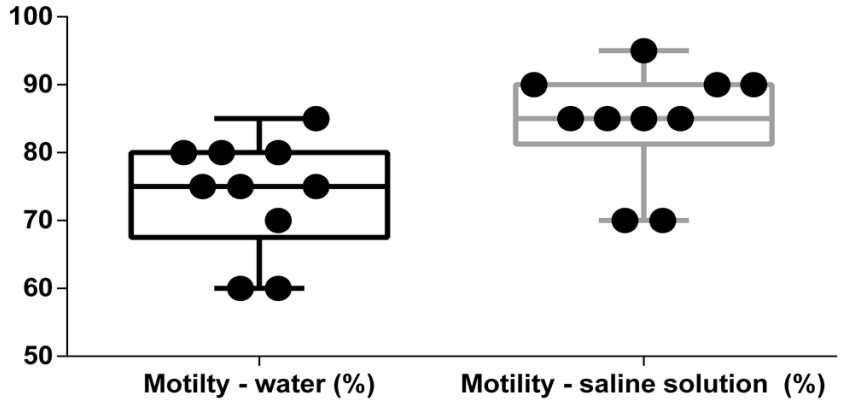

Fig. 3. Minimum, maximum and mean of mobility in water and saline solution

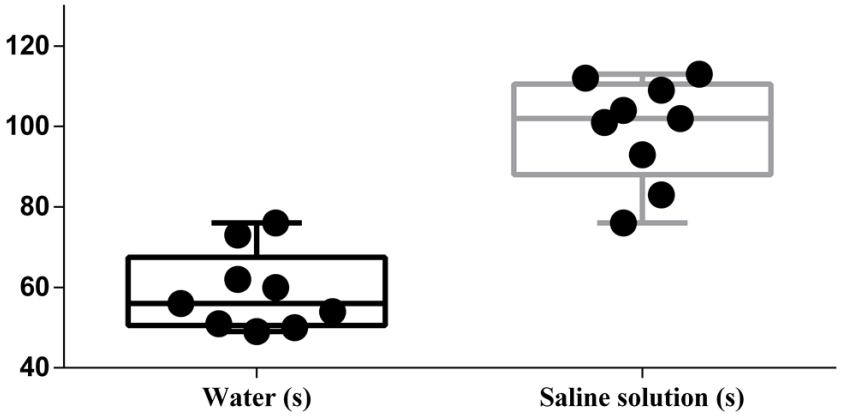

Fig. 4. Minimum, maximum and mean of mobility duration in water and in saline solution

phase and in regeneration phase after the onset of mobility. In the case of cholesterol, there is currently no information on the role it plays in seminal plasma of fish (Billiard *et al*., 1995). Alkaline phosphatase (ALP) activity was averaged at 36.2±0.59 U/L. The ALP concentration is higher than in rainbow trout (8.76 U/L, Ciereszko and Dabrowski, 1994) but smaller that at *H. huso* (76.13 ± 13.56 U/L) and *A. stellatus* (69.05 ± 13.04 IU/L) (Shahsavani *et al*. 2007, 2008).

Figure 3 shows that in the dilution medium (water or saline solution), mobility is between 60-95%, with the lowest mobility being observed in males 2 and 9 (60%, water) and the highest percentage in male 3 (95%, saline solution).

The duration of the mobility (Figure 4) is short, at the beginning the movements observed at the microscope are very energetic but gradually they become slower, oscillatory, till the spermatozoa remain immobile.

When using water as a sperm-activating medium, the duration of the mobility ranges from 49 seconds to 76 seconds, and when 7‰ saline solution was used, the values increase significantly, between 76 seconds and 113 seconds. Thus, the most important change in the duration of mobility is recorded for male 3, where from 56 seconds it reaches 113 seconds and the slowest duration of mobility is registered for male 6, where from 51 seconds it reaches 76 seconds.

By using saline solution as an activating factor, both mobility and mobility duration values are superior to water use. Thus, the use of saline solution increases the fecundity capacity of sperm, leading to a better rate of fecundity and implicitly to superior production. With the increase in mobility and its duration, fecundation stages are favoured, providing the necessary intervals for acrosome reaction and amphimixmia.In Figure 5, there is shown sperm density in a millilitre of semen. In order to determine the density, we

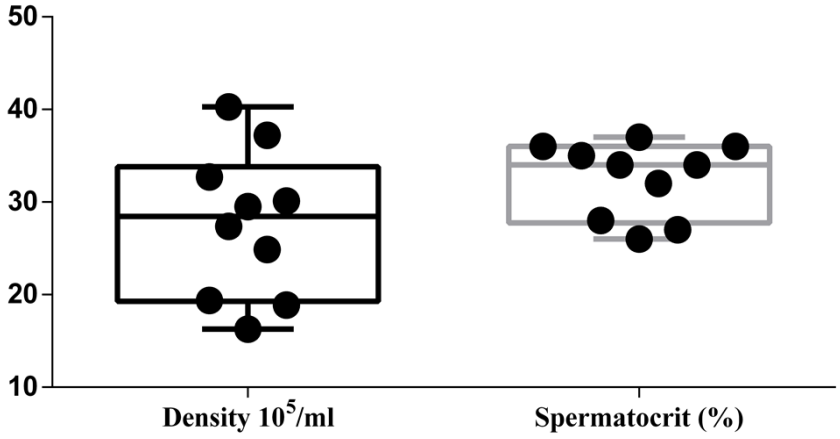

Fig. 5. Minimum, maximum and mean of spermatocrit and density of spermatozoa

used the microscopic examination, determining the spermatozoa count in one millilitre of semen, using the erythrocyte counting method. Also, spermatocrit values are represented, where the values are between 14% (male 3) and 36% (males 2 and 10), with a mean value of 32.5%.

CONCLUSIONS

The knowledge of physical and chemical components of spermatozoa and seminal plasma is mandatory for an objective evaluation of the reproductive ability of salmonids, which leads to a better understanding of the fertilization mechanism.

As a result of researches on semen in brown trout, we noticed that saline solution (NaCl) at a concentration of 7 ‰ compared to water when used as an activator of seminal material is much more effective in activating sperm, prolonging both the duration of their mobility and the percentage of mobile spermatozoa.

The results of this study can be used to make a rigorous selection of high quality reproductive males for the use of semen in the fertilization of roes. Thus, achieving a much higher percentage of hatching roes and much lower mortality in the development of early stages of biological material. Also, from an economic point of view, it will reduce maintenance costs for breeding males, because as a result of the rigorous selection, the number of males from the breeding group will be reduced.

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