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Cell morphological and biochemical studies of the sperm on a rainbow trout brood stock, *Oncorhynchus mykiss* (Walbaum, 1972)

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Abstract. The purpose of these studies consists in the evaluation of the fecundity capacity of the sperm at rainbow male trout correlated with the individual cell-morphological, physical and biochemical characteristics of the sperm, in relation with the survival percentage of the progeny in order to select the best young males. We studied the average individual values and standard deviation, the variability coefficients for the physical and cell morphological parameters, as well as the abnormalities registered at spermatozoa and their frequency. For the biochemical parameters we revealed the individual values, averages and standard deviation, the variation coefficients, the biochemical parameters of the total sperm or the seminal plasma following the fructose concentration, GOT and phosphatase activity. We investigated the effect of the dilution on the fecundity capacity of the seminal material, the evaluation of the registered loses and also the causes of the mortality in fertilized spawns.
Key words: cell morphology, protoplasm, spermatocrit, spectrophotometry.

Resumen. Nuestro propósito consiste en la evaluación de la capacidad fertilizante de la esperma de trucha (*Oncorhynchus mykiss*), teniendo en cuenta los indices individuales citomorfologicos, fisicos y biochimicos de la esperma. Se ha estudiado el efecto de la dilución sobre la capacidad fertilizante del material seminal y la evaluación de las perdidas registradas y las causas de la mortalidad de las huevas fertilizadas.
Palabras clave: morfologia, protoplasma, spermatocrit, spectrofotometria.

Rezumat. Scopul lucrării constă în aprecierea capacităţii fecundante a spermei de la reproducători masculi de păstrăv curcubeu în corelaţie cu indicii citomorfologici, fizici şi biochimici individuali ai spermei, raportat la procentul de supravieţuire al descendenţei în vederea selecţiei reproducătorilor masculi. S-au studiat valorile individuale medii şi deviaţiile standard, coeficienţii de variabilitate pentru parametrii fizici şi citomorfologici precum şi anomaliile înregistrate la spermatozoizi şi frecvenţa lor. Pentru parametrii biochimici s-au urmărit valorile individuale, mediile şi deviaţia standard, coeficienţii de variaţie, parametrii biochimici ai spermei totale sau plasmei seminale, urmărindu-se concentraţia de fructoză, GOT-ul, activitatea fosfatazei. S-a urmărit efectul diluţiei asupra capacităţii fecundante a materialului seminal şi evaluarea pierderilor înregistrate şi a cauzelor mortalităţii icrelor fecundate.
Cuvinte cheie: morfologie, protoplasma, spermatocrit, spectrofotometrie.

Introduction. Culture and artificial or natural reproduction of fish in captivity is suitable for avoiding excessive fishing (Hărșan & Petrescu-Mag 2008), increasing the high quality meat availability (Appelbaum & Arockiaraj 2009; Bura & Szelei 2009), creation of new ornamental strains (Shaddock 2009) and for understanding the animal behaviour (Bourne & Sammons 2008; Bourne & Watson 2009) or animal genetics (Petrescu-Mag & Bourne 2008). There is a tradition on trout farming in Romania, including its artificial reproduction (see Bud et al 2009).

The purpose of these studies consists in the evaluation of the fertilization capacity of the sperm at rainbow male trout correlated with the individual cytomorphological, physical and biochemical aspects of the sperm, correlated with the survival percentage of the progeny in order to select the best young males.

Material and Method. The material was collected by stripping (abdominal massage) from a number of 14 individuals of rainbow trout , aged of four summer, which were in the second cycle of reproduction. Also, the females from which the spawn were provided, have had the same age.

For the physical parameters of the sperm analyzed were the volume and the aspect. The spermatocrit was measured in analogy with the hematocrit (Ht) or packed cell volume (PCV) in capillary tubes, after centrifugation at 5000 rpm, for 5 minutes, and the pH was determined with a digital pH-meter (see Billard & Bretton 1976 for the full protocol).

The cell morphological methods used in examining the seminal material were the microscopic examination, establishing the number of spermatozoa in 1 ml of sperm using the erythrocytes counting method. The viability of the sperm was evaluated by intra-vital staining with eosin-nigrosin (at a temperature of 37^0C, according to Bud 1990).

There were determined the percentage of non-viable spermatozoa, the percentage of spermatozoa with protoplasmic drops and the percentage of malformed spermatozoa (head-tail anomalies, Billard 1986). There were numbering 1 000 spermatozoa for each of the above mentioned parameters and the percentages were established.

Using biochemical methods it was determined by spectrophotometry the concentration of some biochemical constituents for the whole sperm or the seminal plasma. From the raw sperm it was determined the optical density (OD), corresponding to a dilution of 1:400, total protein and protein from the seminal plasma, the fructose concentration, the transaminase and acid phosphatase.

OD of the dissolved sperm was compared to the McFarland scale used in stamping the bacterium density. The sperm was diluted in MMLS medium (1:1000).

The spawn was collected from 10 females, divided in two groups, each with 30,000 spawn: the control one, M, was fertilized with raw sperm and the T group was fertilized with 2.25 ml from the diluted sperm. At the moment of the fertilization a buffer solution of NaCl (250 mOsm, pH = 9) was added.

The effect of the diluted sperm was appreciated at 24 hours, at seven days, at 15 days from the fertilization and at hatching, by numbering and examining 1000 spawns from each group.

There were also examined 100 dead spawn from each group, establishing the cause of mortalities.

The sperm volume, the spermatozoa number and OD of the sperm were positively correlated with the percent of viability, being able to constitute the criteria of examining the quality of the seminal material (see also Billard 1986).

Meaningful correlations have been established between the fructose concentrations and the percent of viability, OD and spermatocrit, the transaminase activity and the phosphatase activity.

The fertilization with diluted sperm of 1:1000 was a success, the registered loses being not a direct cause of the fertilization process.

Results and Discussion. In what concerns the cell morphologic exam from Table 1 it can be noticed that the sperm volume at the 14 samples of sperm taken into study has varied from 3.5 to 17 ml with an average of 8.75±3.80 ml, and the spermatocrit has varied within the interval 9-36% with an average of 18.35%±8.49. The number of spermatozoa/ml of sperm has varied also within a large interval between 10.7 - 33.2 x 10^6/ml, with an average of 21.5±7.07 x 10^6/ml. The coefficient of viability determined in the study reveals a large individual spread.

The percent of viability was over 99.5% in the majority of the investigated samples, while in three samples the viability was under 16%.

Protoplasmic drops have been identified in the spermatozoa at 9 from the 14 samples taken into account, the constant maximum values of frequencies varying between 10 to 28%. The abnormalities of the head (double head, swollen) were also registered but their frequency was in small percentage, between 2 and 3%.

In Table 2 are presented the individual values, the averages and standard deviations as well as the variation coefficient for the biochemical parameters of the total semen or seminal plasma.

Table 1

Physical and cytomorphological parameters determined from the semen

No. samples	*Volume (ml)*	*No. 10^6/ml*	*Spermatocrit (%)*	*Viable coloring parameters %*	*Protoplasmic drops*	*Head abnormalities (%)*
1	3.5	10.2	16	5.6	-	-
2	11.5	28.6	31	92.6	-	-
3	6.9	10.7	17	10.1	1,5	-
4	12.5	17.05	14	99.6	1,0	-
5	6.8	28.0	27	94.7	28,0	2
6	9.6	23.6	10	99.8	10	2
7	5	23	13	15.2	2,5	3
8	4.8	33.2	36	99.9	3	3 double head 5 swollen head
9	12.6	22	26	99.7	-	4 double head
10	17	17.1	17	99.4	5	2 double head
11	10.2	24.5	9	99.7	6	-
12	7.2	17.7	13	92.04	-	5 double head 2 swollen head
13	5.2	14.7	9	99.5	7	-
14	9.8	25.3	19	99.6	-	-
x ± s	8.75±3.80			21.51±7.07		18.35±8,49
CV %	43%			32.8%		46.26%

Table 2

Biochemical parameters determined in the rainbow trout's sperm

No. sample	*Total protein mg/ml sperm*	*Fructose mg/dl sperm*	*GOT μmol/min/ml*	*Fosfatase acid mU/ml plasma*
1.	147.4	0.07	36.48	9.09
2.	134.0	0.86	22.9	18.38
3.	93.8	3.45	18.62	14.94
4.	120.6	2.76	15.73	12.62
5.	150	2.760	27.68	22.22
6.	107.2	2.07	11.32	9.09
7.	99.1	1	17.36	13.93
8.	227.8	0.88	12.58	10.10
9.	152.7	0.55	24.53	19.69
10.	53.6	2.07	13.84	11.11
11.	139.3	2.75	11.32	9.09
12.	91.12	3.8	11.95	9.59
13.	80.4	1.38	9.44	7.57
14.	120.6	3.45	11.57	9.29
x ± s	122.68±41.97	1.99±1.2	22.25±9.21	12.61±4.59
CV %	34.15	60.3	41.39	36.38

There has been noticed a concentration of total protein from the semen between 53.6 – 227.8 g/dl. The fructose concentration from the seminal plasma has varied between 0.07 mg/dl and 3.8 mg/dl plasma.

The GOT level was situated between 9.44 – 48 μmol/min/l while the activity of the acid phosphatase ranged between 9.09 – 22.22 mU/ml.

The spectrophotometric evaluation of the diluted sperm is presented in Table 3, where it is revealed the correlation between the optical density of the samples of diluted semen 1:400 and the McFarland scale, noticing the including of the individual values for DO_{650} in the limits of this scale.

The effect of dilution of the semen on the fecundity capacity of the seminal material is presented in Table 3. Analyzing the data from the two lots (raw semen and the group with diluted semen 1:1000 for the entire period of incubation) it can be noticed that the percentage values have small differences in what concerns loses, with a little advantage for the raw semen lot (raw semen, unfertile spawn) 41.08% in contrast to 47.91%.

The statistic correlations between the different determined parameters and the fertilization capacity (Table 4) gathers the coefficients determined and their statistical meaning, resulting in a distinctive correlation between: the volume of the semen and it's viability, volume of semen – fructose, number of spermatozoa – viability, viability – fructose, viability – phosphates and DO_{650} – spermatocrit.

There were been made microscopic examinations of 1 000 spawn / group, establishing 12% with normal aspect, 39% with visible malformations in the embryo body and 49% embryos were separated from the vitelus.

There were also recorded the losses and the mortalities in the fertilized spawn. At dead spawn during the period embryo-hatching by microscopic exam at 100 pieces we established that 7% were unfertilized spawn, 47% fertilized but not developed and 46% were embryo spawn.

Table 3

Loses registered (%) after the fertilization of 30,000 spawn with dissolved sperm 1:1000 (lot T) and raw semen (lot M), during the entire period of incubation

The registering moment reported to the fecundation	*Loses*	
	Lot T	Lot M
At 24 h (examined 100 spawn)	92 (0.3%)	395(1.3%)
At 7 days (examined 30 spawn)	3 (%)	3 (10%)
At 15 days (until embryo from a total of 30000 spawn)	9969 (33.23%)	9258 (30.86%)
From embryo to hatching	4305 (14.35%)	2668 (8.89%)
Total incubation	14374(47.91%)	13326(41.08%)

The large variability of the morphological traits in salmonids makes difficult to find the certain criteria and the selective methods of appreciating the fertilizing capacity – aspects confirmed also by Billard (1986), Billard & Breton (1976, 1978).

The data obtained are between the limits of the results obtained by the others authors quoted in the literature from the field before regarding the average volume of semen, the spermatozoa number/ ml sperm, the fructose content, the average variability, the acid phosphatase activity.

From statistic analysis of the data results a positive correlation between: the viability percent of the spermatozoa and their number, DO_{650}, the total semen and the fructose concentration from the seminal plasma.

It is important to underline the presence of the "protoplasmic drops" on the tail of the spermatozoa which is to a large extent alike those observed in mammals. This indicates the existence of some immature spermatozoa with a small capacity of fertilization.

The acid phosphatase and GOT are considered the main factors involved in cellular lysis and they are used for the characterization of the quality of the seminal plasma. Having in view the easy way in which we determined DO_{650}, the meaningful relation with the other morphologic or physiologic parameters and the possibility to compare the values with the McFarland scale allows the using of this scale in a rapid evaluation of the quality of the seminal material, in the farms.

Table 4

Correlation coefficients of high rang determined between the physical, cell morphological and biochemical parameters as well as their statistic meaning

Correlated parameters	*r*	*p*
Volume sperm – number spermatozoa	- 0.024	NS
Volume sperm – spermatocrit	+0.092	NS
Volume sperm– % viability	+0.494	p=0.01 ** distinctively significant
Volume sperm – fructose	+0.285	p=0.05 * significant
Volume sperm – DO_{650}	+0.131	NS
Number spermatozoa – % viability	+0.525	p=0.01 **distinctively significant
Number spermatozoa – fructose	+0.017	NS
Number spermatozoa – DO_{650}	+0.395	p=0.05 * significant
% viability – fructose	+0.369	p=0.05 * significant
% viability – DO_{650}	+0.683	p=0.05 * significant
% viability – fosfatase	0.285	p=0.05 * significant
% viability – GOT	+0.015	NS
Fructose - protein	-0.118	NS
Fructose – fosfataze	+0.169	NS
Fructose - GOT	-0.145	NS
DO_{650} – spermatocrit	+0.551	p=0.01 ** distinctly significant
DO_{650} – fosfataze	+0.243	NS
DO_{650} – GOT	+0.219	NS
DO_{650} – fructose	-0.141	NS
GOT – fosfataze	+0.734	p=0.001 ***very significant

Regarding the causes of the loses registered it is to be noticed that the maximum loss were recorded in the fertilized spawn, but with non-developed embryos (53%) for the M lot and 66.3% for the T lot, followed by the fertilized spawn with formed embryo (35%) respectively 25.4%. The percent of non-fertilized spawn is minimum – 12% for the M lot, 8.3% for the T lot. This confirms the success of fertilization by using the diluted sperm, the registered loses not being a direct cause of the fertility process.

The microscopic investigations regarding the aspect of the spawn with embryo, reveals a high percentage (49%) of embryos separated from the vitelus or with others malformations (39%). The same aspects were noticed also at the dead spawn during the period embryo – hatching, resulting 29% embryos apart from the vitelus and 57% embryos with malformations.

We appreciate that the fertilization with the selective seminal material and then dilluted 1:1000 in a conserved medium may have positive effects in production.

Conclusions. Values as the semen volume, the spermatozoa number and OD of the sperm, were significantly correlated to the viability percentage, being able to constitute appreciation criteria of the seminal material's quality.

There were established meaningful correlations between the fructose concentration and the viability percent, between the optical density and spermatocrit, between the transaminase activity and phosphatase activity.

There was successfully made the fertilization with diluted sperm 1:1000, the registered loses not being a direct cause of the fecundity process.

Taking into account the using of the physical, cell morphological and biochemical parameters in the characterization of the quality of the seminal material, the selection of the brood stock based on these criteria becomes possible, in this way ensuring superior percentages in the success of artificial fertilization at rainbow trout.

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