Quantitative Characteristics of Rainbow Trout, *Oncorhynchus Mykiss*, Neo-Males (XX Genotype) and Super-Males (YY Genotype) Sperm

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***Abstract*—**Rainbow trout homogametic males, (XX or YY sex genotype), can be obtained, respectively, through masculinisation of genetic females or induced androgenesis. Aim of this study was to compare reproductive potential of neo-males (XX) and super-males (YY) with heterogametic males (XY). We measured spermatozoa motility parameters, sperm concentration, osmolality and characterized protein profiles in samples of stripped and testicular sperm obtained from XY and YY males, and testicular sperm of XX males. The motile spermatozoa, as measured by both subjective method and CASA, showed no differences between testicular sperm of XX males and stripped sperm of XY and YY males whereas testicular sperm of XY and YY males had significantly lower sperm motility. Result of protein densitometry showed similarities in protein profile between seminal plasma of XY and YY males and testicular fluids of XX males. Testis of XX males showed specific histological structures of cysts consists hypertrophied Sertoli cells.

***Keywords*—**fish, genotype, rainbow trout, sperm.

I. INTRODUCTION

Salmonid fish have a sex-determining system in which genotypes at a single gene or region on largely undifferentiated sex chromosomes determine phenotypic sex [22]. It is well known that exogenous vertebrate steroid hormones applied to immature fish have potential to reverse the phenotypic sex. Androgens will change sex in female direction whereas estrogens will create opposite effect. Induced sex-reversal, namely, masculinisation of XX sex genotypes, is a means to produce monosex all-female populations of fish under XY sex-determining system, including salmonids. Typically, the process of masculinization is induced by oral administration of androgens through feed in the early stages of sex differentiation [15]. Sex reversed females (neo-males) produce sperm upon sexual maturation, which can be used to produce a "treatment-free" generation of all-female XX individuals. All-female trout show faster growth rate because maturation in females starts later than in males; therefore, the production efficiency can be improved when monosex system of production is employed [15].

In the rainbow trout, the sperm ducts of many neo-males do not form properly and the lack or the various stage of sperm duct reduction are an usual after-treatment malformations [7]. It might be caused by difficulties in oral administration of hormones at accurate dose for thousand of fishes at the same time. This imply the way of neo-males exploitation, which are based on sacrificing the fish and subsequent testicular milt collection directly from the malformed testis.

Another manipulation possible in fish is an androgenesis. The key of this process is to induce the development of diploid embryo on the basis of male genetic material. This technique have a great potential in case of banking sperm from endangered fish. In order to inactivate the genome of oocytes X-rays or gamma rays are used [3]. Interruption of the last stage of the first mitotic division leads to the integration of the two copies of chromosomes and to form diploid nuclei, which develops homozygous individual. Induction of ploidity in rainbow trout is usually achieved via pressure shock [2], [3].

In view of the growing aquaculture and the widespread use of "artificial" insemination in fish, it is necessary to monitor the quality of sperm. The basic parameters of sperm quality of fish are the sperm concentration and motility as well as their fertilization ability. Rainbow trout fertilization success was positively correlated with sperm motility parameters [10], [28]. However in other salmonids it has not been confirmed [1], [18], [23]. The research related to sperm quality of homogametic rainbow trout males are scarce and should be enlarged as these form of fish become popular in aquaculture practice.

The objective of this study was to compare the sperm of androgenetic males (males YY) and sex reversed females (males XX) with seperm from the control group (males XY). Information about the quality of gametes obtained from

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homogametic fish might help in the planning of production based on fish obtained by genomic or hormonal manipulation. An important objective of this study was an analysis of the low molecular weight protein composition of seminal plasma to investigate the role of sperm duct in the composition of seminal plasma protein as well as the influence of sex genotype on protein content of seminal plasma.

II. MATERIALS AND METHODS

A. Fish

Fish for the study were produced and held at the Department of Salmonid Research, Inland Fisheries Institute, Rutki, Poland under standard procedures. Control XY males originated from "Rutki" strain.

Androgenetic super-males (YY) originated from a population obtained as described by [2], [3]. In brief, eggs were irradiated with gamma rays (35 000 rads) in order to destroy maternal genome, and subsequently fertilized. At 350 min post fertilization at 10°C, eggs were subjected to hydrostatic pressure shock (7000 psi) for 6 minutes in order to obtain diploid embryo [8]. The resulting androgenetic progeny was composed of either females XX or super-males YY. The fish were reared until sexual maturity. Three years old individuals were used for the study.

Neo-males (XX) were obtained via masculinization using 17-α-methyltestosterone at a dose of 3 mg kg^{-1} of feed administered for 60 days from the beginning of start feeding [4]. Upon sexual maturation, neo-males were identified by the absence of sperm ducts, often accompanied by presence of rudimentary female gonads [4]. Fish for the study were at the age 2+.

In total, for the comparative analysis of sperm quality parameters, sperm samples were collected from 51 XY males and 24 super-males YY (stripped sperm). Additionally, testis were dissected from 8 males XY, 8 super-males YY and 34 neo-males XX, to collect testicular sperm samples (Fig. 1).
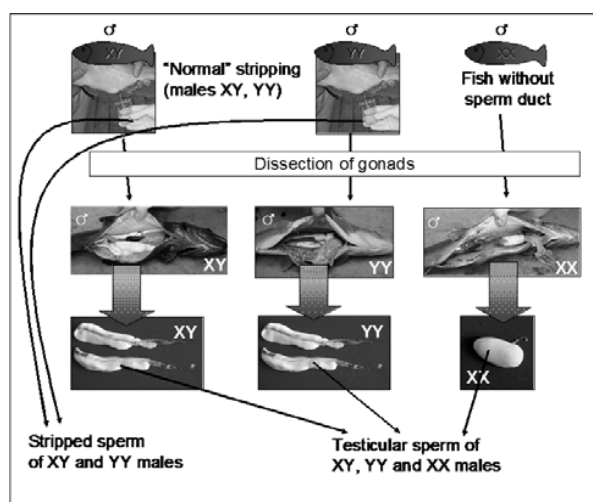

Fig. 1 Schematic of obtaining material for research

B. Histology of gonads

Samples for histology were taken in the middle of the breeding season from mature males. In total, 4 samples of testis were collected from each group of sex genotypes XY, XX, and YY. Excised sections of testis were preserved in Bouin's fluid for 3 days followed by rinsing three times with 70% ethanol, dehydration in ethanol gradients 75, 80, 90, 95, and 100%, and washing three times in xylene. Samples were transferred to liquid paraffin, then embedded in paraffin blocks. Blocks were cut with rotary microtome Leica RM-type 2125, using the transverse or longitudinal sections. Sections of thickness of 5 μm were placed on slides. Prepared samples were subjected to staining with hematoxylin and eosin (HE) or by Mallory. After drying, histological preparations were viewed using light microscopy.

C. Quantitative parameters of sperm quality

Spermatozoa motility was evaluated by subjective method and using CASA system. For subjective method spermatozoa motility parameters were determined under light microscope under the magnification x40. For automatic sperm motility analysis a CASA system manufactured by Hobson's Vision (Hobson Vision Ltd, Derbyshire, UK) was used. Sperm samples were diluted 1:200 (stripped sperm) or 1:400 (testicular sperm) with an activating medium buffer DIA532 [5] before analysis. Immediately after dilution (6s) the aliquot of 0.7 μl was placed on the 12 wells printed microscope slide with (each well had 20μm depth) and sperm movement was recorded under the lens with a magnification of 20x within 12 seconds. Then the tape recordings were analyzed using the Hobson Sperm Tracker. Program settings for the image analysis at x 10 objective magnification were: radius=8.50 mm; predict = off; video = pal; aspect = 1.49; refresh time = 1 s; threshold +11/-50; filter weightings 1=2, 2=3; 3=3; 4=3, image capture rate = 50 Hz. We analyzed the following parameters: VSL - straightlinear velocity of sperm movement (μm s^{-1}), VCL - curvilinear velocity of sperm movement (μm s^{-1}), VAP – total velocity of sperm movement (μm s^{-1}), STR - straitlinearity of sperm movement (%), BCF – beat cross frequency (Hz), ALH - amplitude of lateral head displacement (μm), LIN – linearity of spermatozoa motility (%), MOT - the percentage of motile sperm (%).

Concentration of spermatozoa was calculated by spectrophotometric method [9]. Samples of stripped sperm were diluted 2000 times with a solution of 0.7% NaCl. Samples of testicular sperm were diluted 6000 times. Absorbance was measured at a wavelength of 530 nm. The results were read from a calibration curve prepared for rainbow trout sperm [9].

About 2 ml of sperm samples was centrifuged (10 min, 8 000 x g) to obtain seminal plasma for biochemical analysis. In the case of testicular sperm samples, the testicular fluid remaining after centrifugation of sperm was used for analysis. Total protein content in the seminal plasma and testicular fluid were determined according to Lowry et al. (1951), using bovine serum albumin (BSA) as standard protein. Seminal

plasma and testicular fluids osmolality were measured using the WESCOR ® Vapor Pressure 5520 osmometer and the results were expressed in mOsm kg^{-1}.

Tris-tricine SDS-PAGE was performed in 16% of T 1,3% C2 peptide polyacrylamide gel and 10% T 1, 3% C2 transition polyacrylamide gel [39]. For the comparison of protein bands between seminal plasma and testicular fluids (n=5 in each group) equal amount of protein was applied for electrophoresis and mixed with buffer contained 0.125 M Tris-HCl, 20% glycerol, 4% SDS, 0.3 M DTT with Coomasie Brilliant Blue, pH 6.8. Samples were boiled (100˚C) for 4 min.. Electrophoresis was performed using an anode buffer (+): 200 mm Tris-HCl, pH 8.9 buffer and cathode (-): 100 mM Tris-HCl, 100 mM Tricine, 0.1% SDS. The voltages applied were 30 V - at thickening gel, 60 V - at gel transition, and 80 V until the proteins reached the end of the gel. After separation, the gel was placed for 30 minutes in a fixative (50% methanol + 10% acetic acid) and transferred to a dye (0.025% Coomasie Brilliant Blue). Gels were decolorized in 10% acetic acid within 24 h. Phosphate isomerase (MW 26.625 kDa), myoglobin (MW 16.950 kDa), α-lactoalbumin (MW 14.437 kDa), aprotinin (MW 6.512 kDa), insulin b (MW 3.496 kDa), and baktracin (MW 1.423 kDa; all BioRad, USA) were used as molecular weight standards.

Analysis of electrophoregram densitometry was performed to establish the proportion of the seminal plasma/testicular fluid proteins in each of tested sex genotypes. The study included electrophoreograms stained for protein obtained during the electrophoretic separation. We determined relative optical density (RGO) of all protein bands visible on the gels using software Kodak 1D (Molecular Imagin System, Eastman Kodak Company, USA).

D.Statistical analysis

Data were analysed using GraphPad Prism software (Pizzonia J. Eastman Kodak, New Haven, CT, USA). Data representing percentage of motile spermatozoa were transformed prior statistical analysis with use of arcsine transformation. ANOVA followed by Tukey's post-hoc comparison test was used to evaluate statistically important difference between analyzed groups of males. T-test for paired samples was used to compare protein content and the protein profiles between seminal plasma and testicular fluids.

III. RESULTS

Sexually mature testis of control XY males were built of many lobules (from 100 to 600 μm in diameter) filled with sperm. The walls of lobules were built of connective tissue 10 - 25 μm thick. The outer wall of the testis was 20 - 30 μm thick (Fig. 2a,b). Sperm ducts had multiple membranous structures involved towards the center (Fig. 2c). The interior of the sperm duct was filled with sperm and its walls was covered by epithelial cells (Fig. 2d).
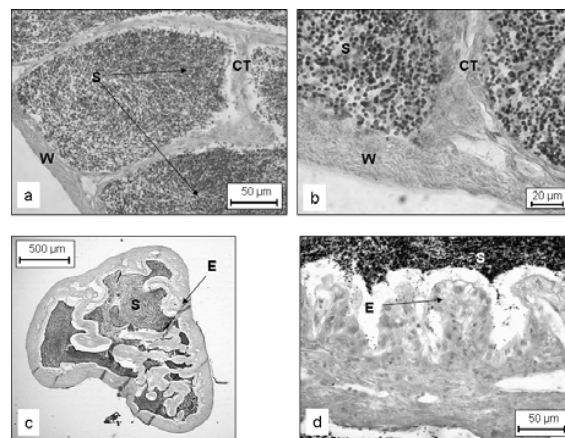

Fig. 2. Histology of testis (a, b) and sperm duct (c, d) of rainbow trout adult male XY at the peak of reproductive season. S - spermatozoa, CT - connective tissue, W - the outer wall of the testis, E – epithelium

In the testis of YY super-males lobules were visible (100 - 300 μm in diameter) filled with sperm. The connective tissue forming the walls of centrilobular cells was visible. The outer wall of the testis had thickness of 20 -40 μm (Fig. 3a,b). Spermatogonial cells were located at distal areas of lobules (Fig. 3b). The center of sperm duct was filled with sperm and its walls lined with epithelial cells (Fig 3c, d).
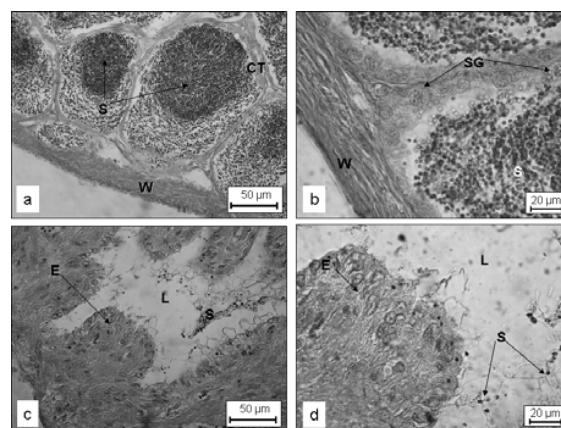

Fig. 3 Histology of testis (a, b) and sperm duct (c, d) of rainbow trout adult super-male YY at the peak of reproductive season. S - spermatozoa, SG - spermatogonia, CT - connective tissue, W - the outer wall of the testis, L – lumen of the sperm duct, E – epithelium

Testis of neo-males XX were built of large lobules (about 1 mm in diameter) located in the centre and smaller lobules (about 50 μm) located in the outer parts of the gonad (Fig. 4c, d, e, f). The thickness of the outer cell wall ranged from 30 to 60 μm.. Cysts of maturing sperm were visible in the connective tissue of the lobules (Fig. 4c, d). Centrilobular cell walls and spermatogonia surrounded by Sertoli cells were observed in the outer parts of the testis (Fig. 4e, f). In the

inner parts of testis, cysts were visible composed of hypertrophied Sertoli cells (Fig 4 e, f).
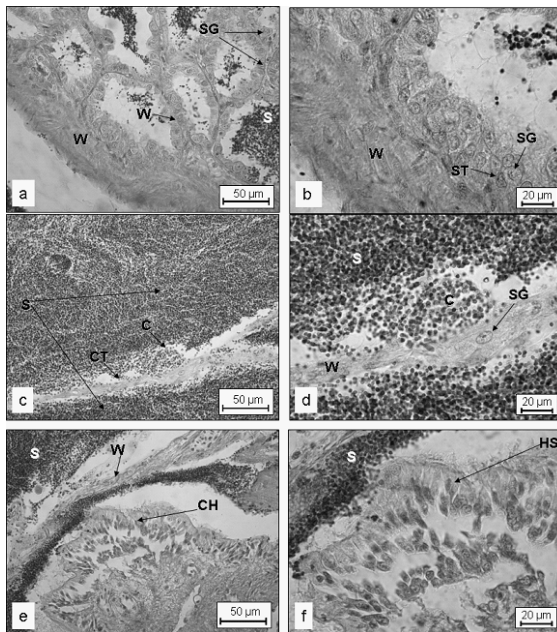

Fig. 4 Histological structures of rainbow trout adult neo-male XX at the peak of reproductive season; a, b - outer part of the testis; c, d, e, and f - the internal part of the testis. S - sperm, SG - spermatogonia, ST - Sertoli cells, CT - connective tissue, C - cyst of maturing sperm, W - the outer wall of the testis, CH - cyst of the hypertrophied Sertoli cells, HS - hypertrophied Sertoli cells.

Sperm motility (MOT) was the highest in samples of the stripped sperm of XY and YY males and the testicular sperm of XX males and it was about 70%. Testicular sperm XY and YY males was characterized by low sperm motility (respectively 48.4 and 33.4%). Sperm curvilinear velocity (VCL) in sperm samples stripped from XY males (average $144.7 \mu m\ s^{-1}$) was significantly higher than in other tested groups (Fig. 5a). VCL of testicular sperm obtained from XX neo-males was on average $127.5 \mu m\ s^{-1}$ and was similar to the values measured for stripped sperm of YY males. The highest strait linear velocity (VSL) and total Speed (VAP was measured for the stripped sperm from the XY and YY. In both groups testicular sperm showed a statistically significant lower value of VSL and VAP ($P<0.01$). The value of STR was significantly higher in testicular sperm compared to stripped one, both in the male XY and YY ($P<0.01$). The BCF did not differ significantly between the groups and ranged from 4.78 Hz (testicular sperm of XX males) to 5.58 Hz (stripped sperm of XY males). ALH was the highest in the stripped sperm of XY males and it was $16.9 \mu m$. The lowest value of ALH was found in stripped and testicular sperm of YY males (respectively 11.1 and $9.1 \mu m$). For both group of males XY and YY, ALH values measured in stripped sperm were significantly higher than the values measured in testicular sperm ($P<0.01$). The linearity of sperm motility (LIN) reached

the highest values in samples of stripped sperm YY males (36.5%). In testicular sperm of XX males this value was close to the value measured for stripped and testicular sperm of XY male (respectively 26.9, 26.8 and 29.2%).
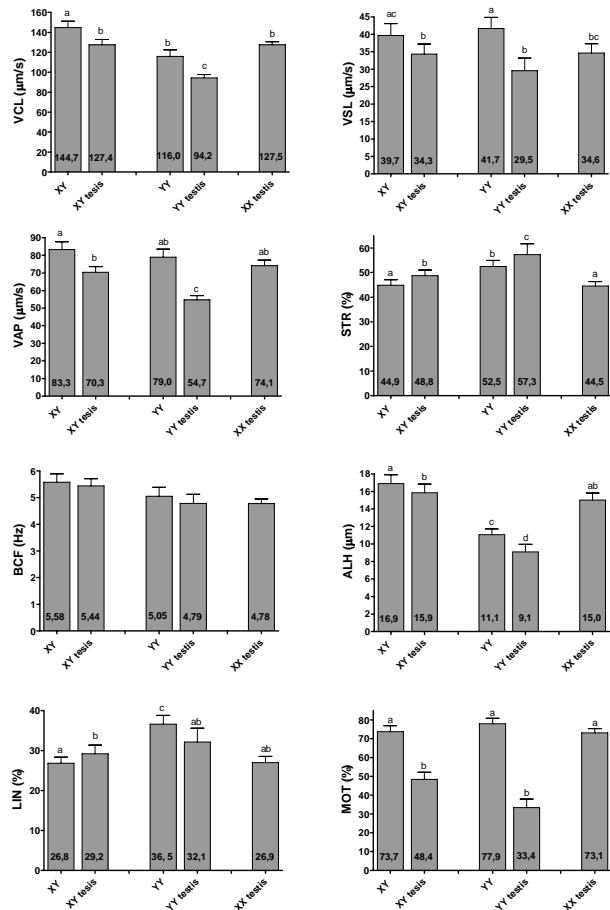

Fig. 5 Motility parameters of stripped and testicular sperm obtained from rainbow trout males with sex genotypes XY, YY, and XX. Different letters indicate statistically important difference between mean values ($P<0.05$)

Similarly to CASA results, sperm motility measured by subjective method was the highest in stripped sperm of XY males and YY super-males, and lowest in testicular sperm samples of XY and YY males. Intermediate values of MOT , not differing significantly from other groups, were found for testicular sperm sample obtained from XX neo-males (Table I).

The lowest spermatozoa concentration was found in stripped sperm from YY super-males ($3.89 \times 10^9\ ml^{-1}$), significantly lower than the value of spermatozoa concentration of stripped sperm from XY males ($6.37 \times 10^9\ ml^{-1}$). The concentration of spermatozoa in all testicular sperm samples was significantly higher and it was over $20 \times 10^9\ ml^{-1}$. (Table I).

Osmolality of seminal plasma and testicular fluid ranged

from 254 to 308 mOsm kg^{-1} . The lowest osmolality was found in samples of YY super-male seminal plasma. The total protein content was lowest in seminal plasma of YY males (0.67g l^{-1}). The highest protein content of sperm was found in testicular plasmas obtained from both, XY and YY, males (respectively 13.36 and 12.29 g l^{-1}; Table I).

TABLE I
BASIC PARAMETERS OF STRIPPED AND TESTICULAR SPERM QUALITY OBTAINED FROM RAINBOW TROUT MALES HAVING SEX GENOTYPES XY, YY, AND XX

	Males XY		Super-males YY		neo-males XX
	Sperm duct n=51	Testis n=8	Sperm duct n=24	Testis n=8	Testis n=34
Motility (%)	62^a ±19	31^b ±15	56^a ±18	29^b ±14	45^{ab} ±25
Sperm concentration (10^9 ml^{-1})	6.37^a ±2.95	22.94^b ±5.22	3.89^c ±1.59	20.94^b ±6.12	21.63^b ±2.65
Osmolality (mOsm kg^{-1})	289^a ±25	301^a ±14	254^b ±31	297^a ±16	308^a ±20
Protein concentration (g l^{-1})	1.14^a ±0.23	13.36^b ±6.13	0.67^c ±0.27	12.29^b ±3.77	7.65^d ±5.05

Average values marked with different letters differ significantly ($P <0.05$) from each other in a line.
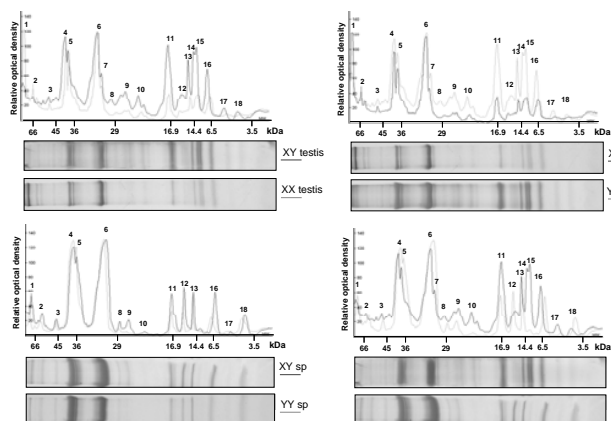

Fig. 6 SDS PAGE electrophoresis of seminal plasma/testicular fluid proteins in denaturing gradient tricine gel. Densitograms of seminal plasma (sp) and testicular fluid (testis).
The X-axis shows the molecular weight.

In the seminal plasma of male XY and YY were relatively more protein with a molecular weight 39.3, 36.7, 31.2 and 4.3 kDa compare to testicular fluids (Table II). Proteins of 31.2 kDa molecular weight were the most abundant in protein profiles in all genotype groups tested. Their content was relatively the highest in the seminal plasma of stripped sperm from XY and YY males, as well as in testicular sperm of XX males. Testicular fluids of XY and YY males showed higher relative optical density of proteins 26.4, 17.0, 15.2, 13.9, and 7.3 kDa.

TABLE II
RELATIVE OPTICAL DENSITY (RGO) OF PROTEIN BANDS IN RAINBOW TROUT SPERM IN DENATURING GRADIENT TRICINE GEL; SEMINAL PLASMA (SP) AND TESTICULAR FLUID (T) WERE OBTAINED FROM MALES OF XY, XX, AND YY SEX GENOTYPES

No Bands	(Da)	XY sp n=5	YY sp n=5	XY t n=5	XX t n=5	YY t n=5
		Relative optical density (±SD)				
1	72 800	0.02^b ±0.01	0.02^b ±0.01	0.01^b ±0.01	0.07^a ±0.02	0.01^b ±0.01
2	63 500	0.01 ±0.01	0.01 ±0.02	+	0.02 ±0.02	+
3	45 300	+	0.01 ±0.02	+	0.01 ±0.02	+
4	39 300	0.19^{ab} ±0.05	0.26^b ±0.09	0.11^a ±0.03	0.14^a ±0.06	0.12^a ±0.03
5	36 700	0.13^a ±0.05	0.14^a ±0.03	0.07^b ±0.03	0.10^{ab} ±0.04	0.06^b ±0.03
6	31 200	0.39^a ±0.05	0.40^a ±0.07	0.21^b ±0.03	0.33^a ±0.04	0.16^b ±0.03
7	30 300	-	-	0.04^b ±0.01	0.07^a ±0.02	0.04^b ±0.02
8	28 800	0.01 ±0.01	0.01 ±0.01	0.02 ±0.01	+	0.01 ±0.02
9	26 400	0.02^a ±0.02	+	0.06^b ±0.02	0.03^a ±0.01	0.08^b ±0.02
10	23 500	+	+	0.02 ±0.01	0.02 ±0.01	0.04 ±0.02
11	17 000	0.07^a ±0.02	0.03^a ±0.03	0.13^b ±0.03	0.06^a ±0.02	0.14^b ±0.04
12	15 800	0.05^a ±0.02	0.04^a ±0.02	0.01^b ±0.01	+	0.01^b ±0.01
13	15 200	0.03^a ±0.01	0.03^a ±0.02	0.07^b ±0.03	0.03^a ±0.01	0.06^b ±0.02
14	14 800	-	-	0.06 ±0.02	0.04 ±0.02	0.03 ±0.03
15	13 900	-	-	0.09^{ab} ±0.03	0.04^a ±0.03	0.10^b ±0.04
16	7 300	0.05^{ab} ±0.03	0.03^a ±0.02	0.08^b ±0.02	0.06^{ab} ±0.01	0.07^b ±0.03
17	5 400	+	+	0.01 ±0.01	+	0.03 ±0.02
18	4 300	0.03^b ±0.01	0.03^b ±0.02	0.01^a ±0.01	+	+

Average values marked with different letters differ significantly ($P <0.05$) from each other in a line. Bands having ROG not exceeding 0.01 are marked as +; absence of band is marked as -.

IV. DISCUSSION

For the first time, we have made a comparative study of sperm quality in all possible sex genotypes in rainbow trout. Results of sperm motility CASA parameters, sperm concentration, protein profile, and histology were collected and compared between the three genotypic group of rainbow trout males (XY, YY and XX). We confirmed that testis of XX males posses some cellular structures composed from hypertrophied Sertoli cells which was not present in XY and YY testis. Our results indicate that testicular sperm obtained from XX males have similar quality to those obtained from

XY and YY males after stripping. Also protein profile of XX testicular sperm was similar to those observed in seminal plasma of XY and YY males. Putting all together we assume that sperm obtained from neo-males testis have similar characteristics to stripped sperm of XY and YY males and higher than testicular sperm of XY and YY males.

Sections of sperm ducts of XY male and YY super-male revealed the existence of a specific folded structure of the epithelium. Sperm duct in teleost fish has secretory function [31], [32]. It regulates, among others, ion concentration and pH of seminal plasma [26]. It was shown in salmonids that the sperm duct plays important role in acquisition of sperm motility potential [35]. The folded structure can increase secretory area of the sperm duct of males XY and YY. This might be related to visible fibrous secretion in sperm duct lumen as was described elsewhere [36].

Histological structures of testis of XY and YY males were similar to testis of Atlantic salmon (*Salmo salar*) and brown trout (*Salmo trutta m. trutta*) [17]. Testis of XX neo-males differed from the testicular histology image of XY and YY males. The cysts of epithelial hyperplasia were found. This tissue, filling lobule, probably consists of hypertrophied Sertoli cells. Similar cellular structures were found in the testis of gladiolus (Xiphophorusmaculatus), subjected to hormonal manipulation [25]. Also in neo-males of rainbow trout such structure was already reported [20]. Image of fibrous secretions on the surface of these cysts might indicate, the excretory function of these structures.

Sperm of most teleost fish is characterized by a straightlinear motility, which is reflected by nearly identical values of VSL and VCL [24]. In rainbow trout, VSL values are usulally much lower than VCL values, which results in low LIN (LIN = VSL/VCLx100%) [13], [14]. In the present study, the highest values of LIN were found in stripped sperm of YY super-male. The values of ALH measured in stripped sperm of YY super-males were similar to those found for stripped sperm of XY rainbow trout (about 8.5 µm) [14]. However, in our work ALH value of samples obtained from XY males were almost twice higher (Fig. 5f). A possible explanation for this divergence of ALH results is the influence of age and a line of fish used for the experiments. References [13] studied males from autumn-spawning strain of (age 3+, whereas in the present study, sperm from fish from spring-spawning strain of age 1+ and 2+ was examined. The low value of the VSL and LIN, combined with high rates of ALH in the case of mammalian sperm indicated hyperactivity [41]. In the case of freshwater fish sperm movement of such nature can be a manifestation of hypoosmotic shock occurred prior to fertilization [38].

The beat cross frequency (BCF) was similar in all groups of males (Fig. 5). The value of this parameter, 4-5 Hz, was similar to the values reported by other researchers [24]. It seems that the value of BCF is a stable feature in rainbow trout sperm movement characteristics and it does not change during the final maturation in spermatic duct.

VCL (curvilinear velocity) is an important factor in fish male reproductive success [19], [28]. VCL in stripped sperm samples of XY males was on average 144.7 µm s^{-1}, which is higher than values reported before for rainbow trout. References [13], [14], [28] reported VCL ranging from 106 to 120 µm s^{-1}. This difference might be a results of using different genetically fish stock or different measurements technique. The lowest values of VCL were found in testicular sperm of XY and YY males. Surprisingly, testicular spermatozoa of XX neo-males had significantly higher value of VCL. This might indicate that while sperm of neo-males are collected from the testis, their structure and physiology are different from that in "normal" males resulting with difference in sperm maturity.

The highest percentage of motile sperm (MOT) was found in the stripped sperm of XY males, YY super-males, and in testicular sperm of XX neo-males (73.7, 77.9 and 73.1%, respectively). MOT in testicular sperm of XY and YY males was significantly lower (48.4 and 33.4%, respectively) Our results are in agreement with earlier observations indicating that testicular sperm of XX neo-males showed motility rates similar to samples stripped from XY males [21]. Taking into account the two most important parameters of sperm, VCL and MOT, it can be concluded that the best quality sperm was stripped sperm from XY males, YY super-males and testicular sperm of XX males, whereas testicular sperm from XY males and YY super-males had significantly poorer quality. This observation imply that physiology of neo-males testis is quite different from that in "normal" males testis. Further study are necessarily to investigate if the XX males gonads have some part of spermatic duct potential in term of inducing maturation of spermatozoa.

The testicular sperm in all groups of males had concentration higher than 20 x 10^9 ml^{-1}. A similar concentration of sperm in the testis of rainbow trout XY and XX males were reported [21]. The concentration of sperm in samples obtained from YY super-males was almost a half less than the concentration of spermatozoa in XY males (on average 3.89 x 10^9 ml^{-1} versus 6.37 x 10^9 ml^{-1}). Sperm concentration values for rainbow trout XY males reported by other researchers, vary from 10 x 10^9 ml^{-1} [12], [30] 3.2 x 10^9 ml^{-1} [27]. The differences in sperm concentration may be an effect of the differences between lines of rainbow trout spawning in autumn or spring [11]. Usually lower than optimal for species sperm concentration is connected with poor quality of males gametes [16]. However in case of YY males their CASA parameters was similar to those from control group except ALH. It seems that low sperm concentration in these fish might be an result of inbreed caused by specificity of technique used for obtaining androgenetic males.

The osmotic pressure of seminal plasma may be an indicator of contamination by urine which might reduce the osmolality (as their native osmolality are lower than 70 mOsm) and consequently decline sperm motility and its fertilizing ability [6], [16], [28], [37]. Our results showed that seminal plasma osmolality had the lowest value in samples

obtained from YY super-males. However, low osmolality values were not accompanied by lower motility which might suggest that it was not a result of urine contamination. Low osmolality was linked with lower protein content in seminal plasma of YY males compared to XY males.

The protein content in seminal plasma of rainbow trout ranges from 0.7 to 2.8 g l^{-1} [40]. In our study, seminal plasma of XY males contained 1.14 g l^{-1} of total protein on average, similarly to the other data [10]. In the case of YY super-males, seminal plasma protein content was much lower (0.7 g l^{-1}). Low protein content in the case of YY males was accompanied by low concentration of spermatozoa. The low sperm concentration and protein content in ejaculate might be a specific feature of YY males.

Testicular sperm protein content was 13.36 g l^{-1} and 12.29 g l^{-1} for males XY and YY, respectively, whereas in the case of XX neo-males –it was lower (7.65 g l^{-1}). Testis of XX neo-males showed the presence of larger lobules, compared with XY and YY males (Fig. 4c). Therefore, they have less connective tissue in the testis than the other groups of males. Since the maceration of the testis may cause a damage to lobules walls, a protein present in the seminal plasma may be partially derived from damaged tissues. This may be more significant for testicular fluid obtained from the samples of XY and YY males than XX males.

References [29] showed that low molecular weight protein of seminal plasma may positively affect sperm motility. Differences in the number of low molecular weight protein bands found in the present study are generally associated with differences between seminal plasma and testicular fluids of XY and YY males. Sperm duct can be a source of some proteins due to fact that some of them are more abundant in seminal plasma than in testicular fluid, as in the case low molecular weight proteins [29]. A positive effect of seminal plasma protein fraction with a molecular weight below 50 kDa on sperm viability were postulated [29]. Perhaps they are a part of conditioning environment of the sperm maturation. Their greater contribution in the seminal plasma protein profile shows a sperm duct as the additional source (outside the testis) of protein synthesis.

Salmonid spermatozoa acquire the ability to move during passing through the sperm duct [34]. Testicular sperm is therefore not finally matured. It is devoid of necessary components of seminal plasma produced in the sperm duct [33]. This might be reflected in the percentage of motile sperm from salmonid testis, lower than in the stripped sperm [18], [21]. Also in our study, sperm motility of testicular samples obtained from XY males and YY super-males was low. However, sperm motility in testicular samples of XX neo-males did not differ significantly from the motility of stripped sperm of XY and YY males. Also other authors reported a lower percentage of motile sperm from testis of XY rainbow trout compared to the motility of testicular sperm of XX males [21]. The difference in the characteristics of testicular sperm between XX neo-males and males having developed sperm duct can be caused by the fact that at the dissection of mature

testis from XY or YY males, large part of finally mature spermatozoa is already released into the sperm duct. Therefore the remaining sperm, although most of which has already completed the process of spermatogenesis (as confirmed by testicular histology), has not yet acquired the full ability to move (lack of final maturation stage).

The sperm motility rate of testicular samples of XX neo-males, as measured by both subjective method and CASA, was similar to sperm motility measured in stripped sperm of XY and YY males. It might be a result of compensatory (for sperm duct) structure found in XX males testis probably derived from hypertrophied Sertoli cells [20]. It is possible that cysts of hypertrophied Sertoli cells can regulate the composition of testicular plasma and synthesize proteins, homologous to that from sperm duct. This is corroborated by the result of protein densitometry showed similarities in protein profile between seminal plasma of XY and YY males and testicular fluids of XX males.

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