

Application of Spermatozoa from Neo-males (XX) to Induce Androgenetic Development in Rainbow Trout (*Oncorhynchus mykiss*)

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In species like rainbow trout (*Oncorhynchus mykiss*) that show male heterogamety (XY), induced androgenesis results in production of females (XX) and males (YY). Morphological and genetic differences between rainbow trout X and Y chromosomes suggest that androgenetic males (YY) may show lower survival than androgenetic females (XX). To test this hypothesis, we produced lines of androgenetic rainbow trout composed of males (YY) and females (XX) and of only females (XX) using spermatozoa from normal males (XY) and neo-males (XX), respectively. Survival rates of the androgenotes were monitored until gonadal differentiation and no significant differences in survival between XX/YY and XX androgenetic rainbow trout lines were observed. Sex-genotypes of the paternal males and androgenetic offspring were confirmed using a rainbow trout Y chromosome-related DNA marker. Histological analysis performed to detect potential disturbances in the gonadal development of the androgenetic XX and YY rainbow trout showed proper development of testes and ovaries in most of the studied individuals. Our results indicated that differences between rainbow trout X and Y chromosomes did not affect survival of the androgenetic offspring. Application of spermatozoa from neo-males to induce androgenetic development may have some potential in rainbow trout aquaculture to produce homozygous females that may be used in breeding programmes and to establish clonal lines.

Key words: androgenesis, gonadal differentiation, rainbow trout, sex chromosomes.

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Androgenesis is a reproductive system in which diploid offspring carry only paternal chromosomes. It is a rare phenomenon observed in a limited number of plant and animal species (MCKONE & HALPERN 2003). Exclusive paternal chromosome inheritance can also be induced intentionally and is known as artificial androgenesis. In fish, androgenetic development is achieved by insemination of the irradiated eggs showing inactivated

nuclear DNA. Paternal chromosomes in the androgenetic haploid zygotes may be duplicated by suppression of the first cleavage accomplished by exposition of the haploid zygotes to temperature or pressure shock (PARSONS & THORGAARD 1984).

Androgenetic individuals have been utilized in selective breeding programs and production of monosexual and clonal fish lines. Androgenotes may also be used in studies concerning recessive

alleles, genetic mapping and genome sequencing. Exclusive paternal inheritance is a valuable tool in sex determination studies (KOMEN & THORGAARD 2007). Androgenesis has also been induced to recover nuclear genomic information from normal and cryopreserved spermatozoa (BABIÁK *et al.* 2002a) and to investigate the effects of the cytoplasm and mitochondria on embryo development (BROWN & THORGAARD 2002). Unfortunately, expression of lethal alleles and side effects of the manipulations performed on the eggs trigger high mortality among androgenetic specimens which is still a limiting factor in the wide application of this reproductive technique. Moreover, survival of androgenetic specimens may be affected by the sex chromosomes. In rainbow trout, males are heterogametic (XY) and the androgenetic progenies are males (YY) and females (XX). Rainbow trout Y chromosome shows a reduced p-arm lacking 5S rDNA arrays observed on the rainbow trout X chromosomes (MORAN *et al.* 1996; OCALEWICZ *et al.* 2007). A rainbow trout sex determining gene has been mapped to the Y chromosome quite recently (YANO *et al.* 2012). Genetic differences between rainbow trout X and Y chromosomes may result in lower survival of the androgenetic males (YY). Furthermore, fish with uniparental chromosomes may display alterations in gonadal differentiation including delay of gonadal development, dysgenesis or even sterility (ZHANG *et al.* 2015). Thus, to assess if sex determination and gonadal differentiation processes affect the efficiency of androgenesis in rainbow trout, stocks of all-female (XX) androgenotes and stocks composed of both males (YY) and females (XX) were generated using spermatozoa from neo-males (XX) and normal males (XY), respectively. Survival rates of the androgenotes were monitored during embryonic development and after hatching. Genetic sex of the paternal individuals and androgenotes was confirmed at the molecular level using Y chromosome related DNA markers and gonadal differentiation was studied histologically.

Material and Methods

This study was approved by the Local Committee on the Ethics of Animal Experiments in Gdańsk, Poland (no. 28/2015).

The experiment was performed in the Department of Salmonid Research, Rutki, Poland, and all rainbow trout gamete donors belonged to the Rutki strain (OCALEWICZ 2002). All individuals were euthanized by means of an overdose of anesthesia (MS-222, Sigma-Aldrich, USA) prior to further analyses.

Gamete collection and inactivation of maternal nuclear genome

Eggs and coelomic fluid were collected from 10 females to separate plastic bowls, whereupon portions of low quality gametes including some damaged and white eggs were discarded. The remaining eggs were pooled and divided into two batches: one was prepared for irradiation ($n=11\,200$) and the other contained eggs for the control treatments of the study ($n=4\,700$). Eggs from both batches were stored in coelomic fluid at 4°C for further treatment. The batch of eggs for the androgenetic treatment (A) was transported in a covered plastic container on ice to the Clinic of Oncology and Radiotherapy, University Clinical Center, Medical University of Gdańsk, Gdańsk, Poland where they were subjected to 360Gy of X-rays emitted by the linear accelerator Clinac 600 (Varian Medical Systems, Palo Alto, CA, USA) (OCALEWICZ *et al.* 2010). To reduce any risk of photoreactivation of the DNA, the irradiated eggs were covered with protective black foil and transported on ice back to the Department of Salmonid Research, Rutki.

Insemination and duplication of the paternal set of chromosomes

The milt was obtained from three normal males (XY) and three sex-reversed males (neo-males) (XX). Sperm of each male/neo-male was used to inseminate about 750 unirradiated eggs and about 1800 irradiated eggs. All eggs were inseminated at a sperm/eggs ratio of 300 000 to 1 in the presence of Billard solution (154 mM NaCl and 1 mM Ca^{2+} , buffered to pH 9.0 with 20 mM Tris + 30 mM glycine) (BILLARD 1992). Inseminated unirradiated eggs constituting the control groups (C) were placed in the incubation baskets in the hatchery. Inseminated irradiated eggs (A) were incubated in 10°C for 5 hours 50 minutes. Then, to double the paternal haploid set of chromosomes, irradiated and activated eggs were subjected to high pressure shock (51.71 MPa) for 4 minutes using TRC-APV electric/hydraulic apparatus (TRC Hydraulics Inc. Dieppe, Canada). After this treatment eggs were also transferred to the incubation baskets. All eggs were incubated in three separate replicates at 6–8°C.

Survival

All experimental groups were meticulously reviewed and counted at the eyed stage (24 days post fertilization, dpf), at hatching (44 dpf) and the swim-up stage, after complete resorption of the yolk-sac (62 dpf). Survival rates of the progeny are presented as means \pm SD. The Statistica software, version 12 (StatSoft, Tulsa, OK, USA) was used

for statistical analysis. Non-parametric Kruskal-Wallis test was chosen to compare the results, considering statistical significance of P value <0.05. Hatched androgenetic individuals were reared for nearly 6 months before further analyses.

Fish sampling

Juvenile androgenotes were sampled on the 162nd day after hatching (dah) to determine their genetic sex and differentiation of the gonadal tissue. After examination of their morphology, gonads were collected for histology and fin clips were sampled for DNA extraction.

Molecular analyses

Nuclear DNA for molecular analyses was extracted from fin tissues of paternal males and all androgenotes that survived up to the histological sampling. Isolation was performed with Genomic Mini AX Tissue Spin (A&A Biotechnology, Gdynia, Poland). Sex genotypes of all tested fish were examined by amplification of rainbow trout Y chromosomes-related DNA sequences (YANO *et al.* 2013). Duplex PCR was performed in the presence of primers for locus 18S rDNA as a positive control (YANO *et al.* 2013). Reaction buffer contained 2.5 ml of 5X Colorless GoTaq® Flexi Buffer (Promega, Madison, WI, USA), 1.5 mM MgCl₂, 0.25 mM of each dNTP (Promega), primers 18S rDNA F and R (0.05 µM each), primers sdY F and R (3 µM each), 7 µl of DNA and 0.3 U of Taq Polymerase (Thermo Fisher Scientific, USA). The reaction was performed in a total volume of 25 µl in a Biometra® T1 Thermocycler. The PCR program involved 3 minutes of initial denaturation at 95°C and 35 cycles (30 seconds of denaturation at 95°C, 30 seconds of annealing at 60°C, 30 seconds of extension at 72°C) followed by the final elongation at 72°C for 3 minutes. To confirm only paternal inheritance, examination of two microsatellite DNA loci: OMM 1381 and OMM 1191 was performed

in strict accordance with the PCR protocols recommended by RODRIGUEZ *et al.* (2003) and SPIES *et al.* (2005), respectively. Homozygosity was estimated in all males used in the experiment and in all obtained viable androgenotes. Products of amplification were separated in 3% agarose gel (Sigma-Aldrich, USA) with addition of ethidium bromide (0.05 mg/ml), and the images were captured with the use of the BioDoc-It™ UV transilluminator (Ultra-Violet Products Ltd, Cambridge, UK).

Histology

Fragments of gonads from ten diploid androgenotes were fixed in Bouin's solution, dehydrated in ethanol, immersed in xylene, embedded in paraffin blocks, and then sliced into 4-5 µm sections with the RM 2155 rotary microtome (LEICA Microsystems, Wetzlar, Germany). Cross-sections of tissues were stained with haematoxylin and eosin (H&E) (ZAWISTOWSKI 1986). The nomenclature of cells and cellular structures of the gonads followed BROWN-PETERSON *et al.* (2011). The analyses were performed with a LEICA DM 3000 light microscope as well as LEICA QWin Pro image processing and analysis software (LEICA Microsystems AG, Heerbrugg, Switzerland).

Results

Survival

Survival rates of the progeny from the control groups were above 84% at the swim-up stage. Survival rates of the androgenetic individuals are presented in Table 1. Differences between survivability among the progeny of all six males used in the experiment were statistically insignificant. After the first feeding, androgenetic groups produced with sperm of males (XY) and neo-males (XX) counted 30 and 31 individuals respectively. Until the 162nd dah when material for histological and

Table 1
Survival rates of androgenetic progeny of the rainbow trout (*Oncorhynchus mykiss*) males (XY) and neo-males (XX) expressed as means ± SD (%)

Sperm donor	Sex genotype	Eyed stage	Hatching	Swim-up stage
Male 1	XY	23.0%±5.85%	6.4%±1.58%	0.5%±0.33%
Male 2	XY	25.6%±7.51%	3.8%±0.74%	0.4%±0.26%
Male 3	XY	13.6%±3.97%	3.8%±0.25%	0.6%±0.30%
Neo-male 1	XX	16.6%±0.40%	4.0%±0.74%	0.5%±0.38%
Neo-male 2	XX	16.3%±4.49%	3.2%±0.48%	0.8%±0.46%
Neo-male 3	XX	19.8%±1.10%	6.3%±2.38%	0.4%±0.62%

molecular analyses was collected, most of the fish had died. Very high mortality exceeding 70% during sex differentiation of manipulated fish resulted in the survival of only three and seven androgenotes, produced with XY and XX semen, respectively.

Molecular analyses

Molecular examination of sex-genotypes in the six males used as gamete donors in this experiment confirmed lack of the Y chromosome in neo-males as well as its presence in the normal males (Fig. 1). Two androgenetic individuals inherited the Y chromosome, whereas Y chromosome-related marker was absent in the other eight examined progenies. These individuals were XX androgenetic females. Examination of microsatellite DNA sequences OMM 1381 and OMM 1191 confirmed only paternal inheritance in all tested androgenotes. Results of amplification showed homozygous genotypes of the manipulated progeny, while males used in this experiment as gamete donors showed heterozygosity in at least one of these *loci*.

Histology

Two of the specimens that survived until histological analysis exhibited body developmental disorder. In comparison to their siblings, both individuals were undersized and showed remarkably higher body depth. These fish were described as dwarf specimens.

Histological examination of the androgenotes confirmed the presence of eight females and two males. Of the eight females, two had gonads with previtellogenic oocytes and a few oogonia. The beginning of ovarian lamellae formation was visible in these fish. The dwarf female presented gonad histology similar to that described above. Two females had gonads with oocytes varying in size, the majority of which were at the final stage of previtellogenesis and with only slightly visible ovarian lamellae. One female had small gonads with a few previtellogenic oocytes and relatively abundant interstitial tissue. The most developed female

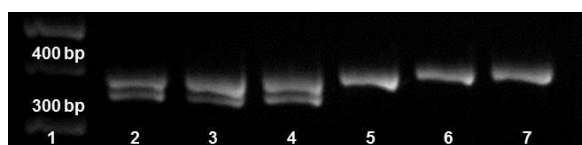


Fig. 1. Sex genotypes of rainbow trout males used in the experiment as gamete donors: lane 1: O'GeneRuler™ 100 bp DNA Ladder (Fermentas, Vilnius, Lithuania), lanes 2-4: sex genotypes of heterogametic males exhibiting presence of both amplification products – 18S and Y-related DNA sequences, lanes 5-7: sex genotypes of neo-males (XX) confirming lack of the Y chromosome.

displayed comparatively large gonads with very distinctive ovarian lamellae and highly diversified oocytes, both in size and development. Single oogonia were also noticeable. One female had atypical gonads with large amounts of interstitial tissue as well as single oocytes in various stages of development (Fig. 2 A, B). The dwarf male had gonads in an early stage of cytological differentiation with spermatogonia visible inside the seminal vesicle. The other male had testes showing the III/IV stage of maturity, with the domination of primary and secondary spermatocytes. Seminal vesicles with cells exhibiting various stages of spermatogenesis and only a few spermatids were visible (Fig. 2 C, D).

Discussion

A traditional inbreeding program is a time and money consuming approach used to provide inbred fish lines. Contrary to parent-offspring mating schemes, artificially induced androgenesis results in producing fully homozygous fish in a single generation. Unfortunately, the efficiency of androgenesis is very low and androgenetic fish have been produced in a very limited number of species (KOMEN & THORGAARD 2007). In the case of salmonids, viable androgenotes have been generated in the rainbow trout (PARSONS & THORGAARD 1984; SCHEERER *et al.* 1991; BABIAK *et al.* 2002a), brook trout (*Salvelinus fontinalis*) (BABIAK *et al.* 2002b; MICHALIK *et al.* 2014), hybrids of the brook trout and Arctic charr (*Salvelinus alpinus*) (OCALWICZ *et al.* 2013) and amago salmon (*Oncorhynchus masou macrostomus*) (KOBAYASHI *et al.* 1994; NAGOYA *et al.* 1996). Yields of the hatched androgenetic DHs vary from less than 1% to 12% (KOMEN & THORGAARD 2007). In the present research, the majority of the androgenetic embryos did not survive up to the hatching stage. This may be due to the expression of the recessive alleles rather than differences between rainbow trout X and Y chromosomes as androgenotes exhibiting XX and YY genotypes did not show increased mortality when compared to the all-female (XX) androgenotes. Results of the present research confirmed what has been observed in the rainbow trout androgenetic haploid embryos (MICHALIK *et al.* 2016). The results show that despite morphological and genetic differences rainbow trout X and Y chromosomes are at the early stage of differentiation and still contain genetic information essential for the proper development of rainbow trout embryos and hatchlings.

The gradual loss of the androgenetic individuals observed from the early developmental stages proved that homozygous offspring with only paternal inheritance suffered from inbreeding de-

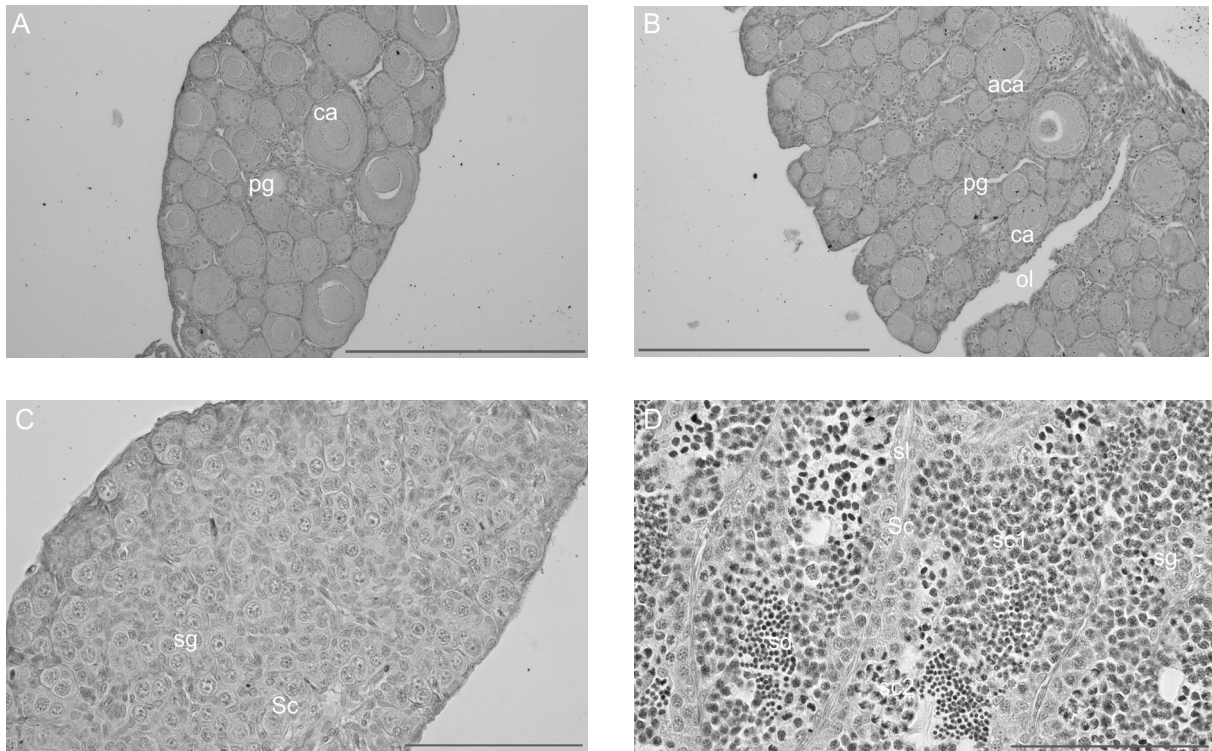


Fig. 2. Histological cross-sections of androgenetic rainbow trout: A – stage II/III of ovary maturity (with no visible ovarian laminae); B – stage II/III of ovary maturity with regular ovarian laminae; C – stage II of testis maturity (immediately after cytological differentiation); D – stage III/IV of testis maturity. Descriptions used: aca – atretic cortical alveolar oocyte, ca – cortical alveolar oocyte, og – oogonium, ol – ovarian laminae, pg – primary growth oocyte, sg – spermatogonia, Sc – Sertoli cell, sc1 – primary spermatocytes, sc2 – secondary spermatocytes, sd – spermatids, sl – wall of seminal vesicles (interstitial tissue), sz – spermatozoa. A, B – bars = 500 μ m; C, D – bars = 100 μ m.

pression. A high rate of individuals with external malformations is observed among fish from the inbred strains (WINEMILLER & TAYLOR 1982). In the case of two androgenotes, one female and one male, stunted growth was observed. Dwarf individuals have also been observed among androgenetic offspring produced within previously performed experiments in the rainbow trout (OCALWICZ *et al.* 2010) and the brook trout \times Arctic charr hybrids (OCALWICZ *et al.* 2013). Although the dwarf-type males might be related to the sneaker male's phenotype (ISELY & GRABOWSKI 2004), the presence of a dwarf female (not observed in the wild) indicates a genome manipulation-related source of these individuals. The recessive loss of some of the functional alleles may cause stunted growth and body deformations. Nonetheless, high pressure shock applied to eggs has also been proved to induce developmental disorders during early development in fish (YAMAHA *et al.* 2002).

Other biological processes including sexual differentiation, gonadal development, growth, maturation and gamete production may also be affected by the expression of the recessive alleles in the androgenetic fish. In androgenetic and gynogenetic individuals, expression of the recessive genes connected with sex differentiation may result in im-

paired gonadal development including gonadal dysgenesis or even sterility (BONGERS & ZANDIEH-DOULABI 1999; ZHANG *et al.* 2015). However, alterations in gonads are more frequently observed in females, whose reproduction is a very complex process (KELLER & WALLER 2002). In the present research altered gonadal development was observed in two females and included a high proportion of the interstitial tissue as well as few atretic cortical alveolar oocytes. Ovaries with an excess of connective tissue and underdeveloped oocytes were found in the gynogenetic carp (*Cyprinus carpio*) and coho salmon (*Oncorhynchus kisutch*) (NAGY *et al.* 1978; PIFERRER *et al.* 1994). Histological analysis confirmed delayed development of gonads in the gynogenetic Atlantic cod (*Gadus morhua*) (WHITEHEAD *et al.* 2012). All androgenetic males studied here show proper gonadal development, which is in corroboration with the literature stating that the YY rainbow trout males exhibit gonads similar to the gonads of XY males (KOWALSKI *et al.* 2011).

Application of neo-male spermatozoa to induce androgenetic development results in the production of all-androgenetic female offspring. Fully homozygous, androgenetic females may be used for the breeding programmes. Eggs from the an-

drogenetic females activated by UV-irradiated spermatozoa and subjected to pressure or thermal shock to inhibit the first cleavage in the zygote would develop into clonal individuals.

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