**PRODUCTION OF TRIPLOID, DOUBLED HAPLOID (DH)
AND MEIOGYNOGENETIC BROOK TROUT
(*SALVELINUS FONTINALIS*) – EFFICIENCY AND DEVELOPMENT
OF BODY DEFORMITIES***

Krzysztof Jagiełło¹, Marcin Polonis¹, Ligia Panasiak¹, Stefan Dobosz², Konrad Ocalewicz^{1*}

¹Faculty of Oceanography and Geography, Institute of Oceanography, Department of Marine Biology and Ecology, University of Gdańsk, Al. M. Piłsudskiego 46, 81-378 Gdynia, Poland

²Department of Salmonid Research, Inland Fisheries Institute in Olsztyn, Rutki, 83-330 Żukowo, Poland

*Corresponding author: konrad.ocalewicz@ug.edu.pl

Abstract

In the present research we produced triploid, mitogynogenetic (doubled haploid; DH) and meiogynogenetic brook trout (*Salvelinus fontinalis*) to examine efficiency of these technologies and potential susceptibility of chromosome set manipulated individuals for the spinal disorders. Triploidy was induced by shocking (High Hydrostatic Pressure – HHP) of fertilized eggs 30 min. after insemination. In turn, gynogenetic development was induced by activation of eggs with UV-irradiated sperm. Activated eggs were then exposed to HHP shock applied 30 and 420 minutes after insemination to provide meiogynogenotes and gynogenetic DHs, respectively. When compared to non-manipulated diploids, the highest survival rates were observed among triploid brook trout while DHs showed the highest mortality. Malformation rates in the diploid larvae from the control groups did not exceed 7.0% while percentage of malformed triploid individuals equaled 19.1%. Drastically increased number of deformed larvae (> 30%) was observed in both DH and meiogynogenetic individuals. Intensification of kyphosis and scoliosis was clearly demonstrated in the gynogenetic and triploid brook trout. Genetic factors such as increased number of sets of chromosomes in triploids and expression of lethal alleles in the gynogenetic fish plus side effects of HHP shock utilized for retention of the second polar body or inhibition of the first cell cleavage when induced triploid and gynogenetic development have been discussed to affect survival rates and prevalence for the skeletal deformities in the chromosome set manipulated brook trout.

Key words: brook trout, gynogenesis, triploids, deformities, scoliosis

Chromosome set manipulations are reproductive bio-techniques that include radiation-induced inactivation of DNA from gametes and application of chemical

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or physical shocks for suppression of the cell divisions. Temperature or high hydrostatic pressure (HHP) shock applied to fish eggs shortly after activation with normal spermatozoa disturbs second meiotic division and prevents release of the 2nd polar body, which results in development of the triploid embryos. Induced development of haploid embryos with exclusive maternal (gynogenesis) or paternal (androgenesis) chromosomes requires activation of eggs with irradiated spermatozoa or irradiation of eggs before fertilization, respectively. Shock applied to eggs early after activation with UV-irradiated sperm results in retention of the 2nd polar body and development of heterozygous diploid meiogynogenotes (Pandian and Koteeswaran, 1998). Duplication of maternal or paternal chromosomes and production of mitogynogenetic and androgenetic individuals called also doubled haploids (DHs) may be achieved by exposition of the haploid embryos to the shock later, around prophase of the 1st mitosis that prevents 1st cell cleavage. Gynogenesis, androgenesis and triploidization have been induced in many laboratory and farm fishes (Pandian and Koteeswaran, 1998; Komen and Thorgaard, 2007; Piferrer et al., 2009). Gynogenetic and androgenetic development of haploid zebrafish, medaka or rainbow trout has been induced to study phenotypic consequences of the recessive alleles (Corley-Smith et al., 1996; Araki et al., 2001; Wiellette et al., 2004), to evaluate impact of sex chromosomes on the early ontogeny (Michalik et al., 2016) and to provide haploid stem cells (Yi et al., 2009). Application of haploid and doubled haploid fish improves *de novo* assembly of the fish whole-genome sequences sequenced using next-generation sequencing approach (Ji et al., 2012; Iwasaki et al., 2016). DHs are used in the selective breeding programs and development of all-female or all-male fish stocks (Dunham, 2004). Gynogenetic and androgenetic reproduction of DHs enables generation of fish clonal lines that have been established in several aquaculture and model fish species (Morishima et al., 2002; Hou et al., 2015; Jagiello et al., 2017). Androgenesis allows recovery of gene pools of valuable fish stocks, populations or even species from the cryopreserved sperm (Babiak et al., 2002). In turn, in fish triploid females are reproductively sterile and their growth and flesh quality are not decreased by the sexual maturation (Maxime, 2008). Sterile triploid fish are not able to interact with the wild stocks of fish when escaped from fish farms or introduced to waters for the recreational purpose (Piferrer et al., 2009; Koenig et al., 2011). Triploidization has been used for sterilization of the transgenic fish (Razak et al., 1999; Nam et al., 2004).

Unfortunately, individuals provided in the course of triploidization, gynogenesis and androgenesis may show reduced survivability (Pandian and Koteeswaran, 1998; Komen and Thorgaard, 2007). Moreover, increased incidences of skeletal deformations are more frequently observed among chromosome set manipulated fish than their non-manipulated siblings from the control groups (Paschos et al., 2001; Sadler et al., 2001; Opstad et al., 2013; Jagiello et al., 2017) (Table 1). Although varied body deformations are observed in fish produced within chromosome set manipulation techniques, no clear pattern of distribution of body disorders among triploids, androgenetic, meio- and mitogynogenetic (DH) specimens has been exhibited (Table 2). Furthermore, the same reproductive manipulations applied to different fish species usually result in different skeletal deformities (Table 2). Fish with body deformations exhibit difficulties in swimming performance and intake of food, which in turn

decrease their growth rate and survivability (Boglione et al., 2013). As production of sterile individuals, monosex stocks of fish and development of breeding programs for aquaculture demand application of chromosome set manipulations, it is important to study ratio of malformed offspring and types of deformations among triploids and specimens with exclusive maternal or paternal chromosomes.

Table 1. Occurrence of deformed specimens among fish produced with chromosome set manipulations (A – androgenesis, G_M – meiogynogenesis, G_{DH} – mitogynogenesis, 3n – triploidization) and their control siblings (selected examples)

Species	Manipulation	Percentages of deformed specimens in experimental group (%)	Percentages of deformed specimens in control group (%)	Literature
<i>Carassius auratus</i>	A	100	–	Paschos et al., 2001
	G _M and G _{DH}	40–60		
<i>Gadus morhua</i> L.	3n	72	42	Opstad et al., 2013
<i>Odontesthes bonariensis</i>	3n	13.89–33.33	0–3.19	Strüssmann et al., 1993
<i>Oncorhynchus mykiss</i>	A	22.2	–	Ocalewicz et al., 2010
	3n	48 ^a 15 ^b	10	Weber et al., 2014
	G _M	4.09–8.85 ^c	0	Jagiello et al., 2018
<i>Oreochromis mossambicus</i>	G _M	16±4 – 20±6	1±1	Varadaraj, 1990
	3n	13±5	2±1	
		7–23	2–6	
<i>Salmo salar</i>	3n	9.1–28	1.5–13.6	O’Flynn et al., 1997
		65	20	Sadler et al., 2001
		3.0	2.66	Cotter et al., 2002
		2–4 ^d	1–2 ^d	Fjellidal and Hansen, 2010
		30–35 ^e	8–12 ^e	
		2.41–3.02 ^d	0.66–0.76 ^d	Fraser et al., 2013
		42.7–48.9 ^e	22.0–24.4 ^e	
<i>Salmo trutta</i> m. <i>fario</i>	G _M	36.1–36.8	13.6	Jagiello et al., 2017
<i>Salvelinus fontinalis</i>	3n	1.0±1.5	1.7±0.4	Galbreath and Samples, 2000
		2.3±4.6	0.9±0.9	
		1.9±1.8	0.3±0.5	
<i>Sander lucioperca</i>	3n	(5.2±1.4, 7±2.5, 9.6±9, 35.5±33.5 to 74.1±0) ^f	1.0	Blecha et al., 2016

^aTriploidization by application of physical shock, ^bTriploidization by mating tetraploid and diploid individuals, ^cSecond round of gynogenesis – malformation rates in fish from clonal lines, ^dMalformation observed by naked eye or examined by hand, ^eMalformation developed by X-ray examination, ^fDepending on the shock conditions.

Table 2. Types of deformities observed in fish produced in the course of chromosome set manipulations (A – androgenesis, G_M – meiogynogenesis, G_{DH} – mitogynogenesis, 3n – triploidization) (selected examples)

Species	Manipulation	Deformation	Literature
<i>Carasius auratus</i>	A	Deformation of the head, the yolk sac and the tail	Paschos et al., 2001
	G _M and G _{DH}	Deformation of the head, the yolk sac and the tail	
<i>Gadus morhua</i> L.	3n	Lordosis, neck deformation	Opstad et al., 2013
<i>Odontesthes bonariensis</i>	3n	Ocular malformation, dilation of the pericardial cavity, bulges in the head, and curling or bending of the body	Strüssmann et al., 1993
<i>Oncorhynchus mykiss</i>	3n	Macrocephalia, lordosis, twisted body	Solar et al., 1984
	A	Body shortening	Ocalewicz et al., 2010
	3n	Vertebral compression	Weber et al., 2014
	G _M	Emaciation, skeletal curvature (scoliosis kyphosis, lordosis) head deformation	Jagiello et al., 2018
<i>Oreochromis mossambicus</i>	G _M	Different body malformations in jaws, eyes, caudal region and megaloccephaly	Varadaraj, 1990
	3n	Short curved tail and edematous body	Varadaraj and Pandian, 1990
<i>Salmo salar</i>	3n	Jaw and body (scoliosis and lordosis) deformities	O'Flynn et al., 1997
		Skeletal deformity, including jaw deformities, short opercula, gill filament deformity syndrome and non-cranial deformities	Sadler et al., 2001
		Eye cataract Hump-back type malformation	Cotter et al., 2002
		Vertebral deformations	Fjellidal and Hansen, 2010
<i>Salmo trutta m. fario</i>	G _M	Spinal deformations (lordosis, kyphosis, scoliosis)	Jagiello et al., 2017
<i>Salvelinus fontinalis</i>	3n	Scoliosis, lordosis, spiral larvae, Siamese twin, double-headed larvae	Galbreath and Samples, 2000

Brook trout (*Salvelinus fontinalis*) is a salmonid fish species originating from North America and introduced to Europe in the 19th century. Brook trout is easily adaptable to the aquaculture conditions including recirculating aquaculture systems. In Europe, brook trout is produced under aquaculture conditions in Poland, the Czech Republic, Germany and Austria, among others. Its hybrids with Arctic charr (*Salvelinus alpinus*) are highly demanded by some European markets. Brook trout is not as susceptible to viral hemorrhagic septicemia (VHS) as rainbow trout (Dorson

et al., 1994), which is an advantage in regions where outbreaks of VHS limit rainbow trout aquaculture production. Though, maturation of males before reaching the market size is the biggest issue in the successful development of brook trout aquaculture (McCormick and Naiman, 1984; Boulanger, 1991). As production of all-female and sterile stocks may solve problem of early maturation, gynogenesis and triploidization of *S. fontinalis* are of interests to the scientists and fish farmers. Even though, information concerning efficiency of chromosomes set manipulations and prevalence of genome manipulated brook trout for malformations is limited. Thus, the main goal of the present research was to induce development of triploid, doubled haploid (DH) and meiogynogenetic brook trout and analyze their survival and susceptibility for the body deformities.

Material and methods

This study was carried out in strict accordance with the recommendations in the Polish Act of 21 January 2005 on Animal Experiments (Dz. U. of 2005 No 33, item 289). This article does not contain any studies with human participants performed by any of the authors.

Broodstocks and Gamete Collection

The experiment was conducted on 7 November 2017. Gamete donors came from the brook trout (*Salvelinus fontinalis*) and the brown trout (*Salmo trutta* m. *fario*) broodstocks kept in the Department of Salmonid Research, Inland Fisheries Institute in Olsztyn, Rutki, Poland. Four-year-old fish were reared in the concrete rotational and rectangular tanks supplied with the river water. Temperature of water ranged from 4 to 6°C. Fish were fed with the broodstock feed (6 mm pellets) and the feeding was stopped a week before gamete stripping. Eggs from five brook trout females (SF_{f1-5}) were collected and kept separately in the 2 liter plastic bowls. Milt from two four-year-old brown trout (ST_{f1} , ST_{f2}) and one brook trout (SF_m) males was collected into the separate 100 ml plastic containers. Sample of sperm (1 μ l) was mixed with sperm activating medium (SAM) (1 mM $CaCl_2$, 20 mM Tris, 30 mM glycine, 125 mM NaCl pH 9.0) (Billard, 1977) (199 μ l) and motility of the spermatozoa was confirmed under the microscope (Nikon Eclipse E 2000). Milt was stored at +4°C for the further use.

Experiment 1. Triploidization

Approximately 1400 eggs from three SF_{f3-5} females were divided into two equal batches to provide triploids (3N) and control diploid (C_{3n}) specimens. Eggs from both batches of brook trout eggs were inseminated with the non-irradiated brook trout sperm (ca. 0.5 ml) in the presence of SAM (ca. 35 ml). Inseminated eggs were thoroughly washed with the hatchery water. To induce triploid development, activated eggs incubated in water at 10°C were subjected to HHP shock (9500 psi/5 min) applied 30 minutes after insemination using TRC-APV electric/hydraulic device (TRC Hydraulics Inc. Dieppe, Canada).

Experiment 2. Gynogenesis

Inactivation of sperm DNA by UV light

For gynogenetic activation of the brook trout eggs we used sperm from brown trout (*Salmo trutta*) in order to eliminate possibility of contamination with the non-inactivated chromosomes (Jagiello et al., 2017). For UV inactivation a standard protocol including dilution of sperm in the rainbow trout seminal plasma and UV irradiation was used with slight modifications (Polonis et al., 2018). To provide seminal plasma, about 50 ml of freshly collected sperm was divided into 15 ml tubes and centrifuged (4000 rpm for 5 min). After centrifugation, seminal plasma was collected, placed in the plastic container (about 25 ml) and frozen (-20°C) to damage any functional spermatozoa. A 0.375 ml portion of sperm was diluted in 15 ml of seminal plasma in a 60 ml glass beaker (50 mm diameter, 30 mm height). A beaker with 15.375 ml of diluted sperm (depth of diluted sperm: 7.8 mm) was placed on a magnetic stirrer and exposed to the UV-C light source (Phillips TUV 30-Watt UV bulb) for 12 min. The distance between the surface of the magnetic stirrer and the UV lamp was 20 cm. UV intensity and UV dose equaled $2.075 \mu\text{W}/\text{cm}^2$ and $14.94 \text{ J}/\text{m}^2$, respectively. During the irradiation, diluted sperm was mixed using the magnetic stirrer. Immediately after UV exposure, motility of the irradiated spermatozoa was proved after activation with SAM under the microscope.

Induction of gynogenetic development

Eggs from two females (SF_{f1} and SF_{f2}) were used to produce gynogenetic specimens. Number of eggs obtained from SF_{f1} and SF_{f2} females equaled approximately 2400 and 2800, respectively. To induce mitotic gynogenesis (DH), meiotic gynogenesis (G) and to provide normal diploid specimens (control, C), eggs from SF_{f1} and SF_{f2} females were divided into three batches containing 1100 (DH_1), 600 (G_1), 700 (C_1) eggs and 1300 (DH_2), 700 (G_2), 800 (C_2) eggs, respectively. To induce gynogenetic development, portions of diluted and UV-irradiated sperm of brown trout were poured over the DH and G batches of eggs and activated by SAM. Activated eggs were thoroughly washed with the hatchery water and incubated for the further treatment at 10°C . About 100 eggs activated with UV-irradiated spermatozoa from each female were left to develop as haploid controls. To recover diploid state in the remaining gynogenetically activated eggs, HHP shock (9500 psi for 5 min) was applied to activated eggs incubated at 10°C 30 and 420 minutes after activation for meiotic (G) and mitotic (DH) gynogenesis, respectively (Ocalewicz et al., 2013) using TRC-APV electric/hydraulic device. To provide control groups, batches of brook trout eggs were inseminated with the non-irradiated brook trout semen in the presence of SAM.

Survival rates and lethal malformations

Eggs from presumed triploid and gynogenetic variants and their controls were reared in the salmonid egg vertical incubators at $6-8^{\circ}\text{C}$ in three replicates. Damaged (white) eggs were removed within first 24 hours after insemination. Dead and live embryos and larvae were counted at the eyed-stage, at hatching, at the swimming stage and survival rates were calculated. Until the swimming stage all dead larvae

(malformed and normal) from all examined groups were collected and placed in the absolute ethanol. Month after the swimming stage all of the malformed fish were sedated and placed in the absolute ethanol. Preserved larvae were then examined under a binocular microscope (Nikon SMZ16) and photographed using a digital camera Opta-Tech 5 MPIS and OptaView-IS software (Opta-Tech) to assess morphology and to evaluate body deformations. A total of 588 dead larvae were examined ($C_{3n} - 65$, $3n - 180$, $C_1 - 92$, $G_1 - 145$, $DH_1 - 78$, $C_2 - 20$, $G_2 - 8$).

Molecular verification of gynogenesis

Nuclear DNA was extracted from the fin tissue of the parental individuals and from the tails of their normal and gynogenetic progenies (10 specimens from each group) using Genomic Mini AX Tissue (A&A Biotechnology). Three polymorphic microsatellite markers (Sfo-12, Sfo-226, Sfo-D75) (Angers et al., 1995; Perry et al., 2005) were used to examine gamete donors and larvae from the control and gynogenetic groups. Genetic sex of the gynogenetic offspring was evaluated by application of Y-chromosome related DNA marker (sdY) (Yano et al., 2013).

PCR reactions were conducted using the Applied Biosystems SimpliAmp Thermal Cycler and GoTaq[®] Hot Start Green Master Mix (Promega) reaction mixtures. The reaction conditions were: initial denaturation at 94°C for 4 min, 30 cycles of 94°C for 30 s, 56–61°C for 30 s and 72°C for 30 s, final elongation at 72°C for 10 min. PCR products were separated on 2.5% agarose gel (Sigma), stained with ethidium bromide (0.05 mg/ml) and visualized under a UV transilluminator, Vilber Lourmat ECX-20.M. Photos were taken with the Cannon PowerShot G16 digital camera.

Cytogenetic analysis

Brook trout from the broodstock reared in the Department of Salmonid Research have 84 chromosomes (Ocalewicz et al., 2004). Cytogenetic studies were carried out on ten triploids, ten gynogenetic specimens and 8 individuals from the diploid control groups. Somatic metaphase chromosomes were prepared from the cephalic kidney cells according to method described by Ocalewicz et al. (2013).

Statistical analysis

For the statistical analysis non-parametric tests were used. In Experiment 1, significance of differences in survival of triploids and diploids was examined using U Mann-Whitney test. In turn, Kruskal-Wallis test was used to estimate significance of differences in survivability of fish from meio-, mitogynogenetic and control groups in the Experiment 2. Value of $P < 0.05$ was considered statistically significant for both tests. All calculations were performed with IBM SPSS Statistic Viewer software.

Results

Survival rates

In Experiment 1, survival rates of fish from triploid group equaled $85.2 \pm 1.9\%$, $75.4 \pm 2.8\%$ and $74.84 \pm 2.9\%$ at the eyed stage, at hatching and at the swim-up stage,

respectively. Diploid brook trout from the control group when compared to triploids exhibited higher survivability ($87.3 \pm 1.3\%$, $80.8 \pm 2.9\%$ and $80.2 \pm 3.3\%$, respectively) but the differences were not statistically significant.

Figure 1. Survival rates of normal diploid (C), meiogynogenetic (G) and mitogynogenetic Doubled Haploid (DH) brook trout embryos and larvae

In Experiment 2, survivability of diploid progenies of SF_{f1} from the control group was very high during embryogenesis ($97.5 \pm 0.2\%$) and after hatching ($95.9 \pm 0.1\%$) (Figure 1). In turn, progenies of SF_{f2} showed substantially decreased survivability during embryogenesis ($33.2 \pm 11.1\%$), at hatching ($30.1 \pm 9.9\%$) and at swimming stage ($29.9 \pm 10.4\%$) (Figure 1). Gynogenetic haploid progenies of SF_{f1} showed survival rate of 68.9% at the eyed stage while entire haploid offspring of female SF_{f2} died before that stage. None of the haploid individuals survived till the hatching stage. Mortality among gynogenetic embryos and larvae was significantly increased in DH (mitogynogenesis) groups. Survival of mitogynogenetic offspring of SF_{f1} female before hatching, just after hatching and at swimming stage equaled $45.6 \pm 2.7\%$, $12.1 \pm 1.5\%$ and $8.6 \pm 1.8\%$, respectively (Figure 1). Gynogenetic DHs that were developing in eggs from SF_{f2} female died before hatching (survival during embryogenesis was $0.24 \pm 0.42\%$). Similar trend was observed among meiogynogenetic offspring of two females that were egg donors for the research. Meiogynogenetic progenies of SF_{f1} female showed significantly better survival at each examined stage ($72.2 \pm 2.5\%$, $45.5 \pm 3.6\%$ and $42.6 \pm 5.8\%$) than meiogynogenetic offspring of SF_{f2} female ($2.77 \pm 0.5\%$, $1.1 \pm 0.6\%$ and $1.1 \pm 0.6\%$) (Figure 1). Differences in survival of both normal and gynogenetic offspring of the two egg donors were significant. Survival of meiogynogenetic trout was substantially higher than for gynogenetic Doubled Haploids, however both exhibited significantly higher mortality than normal diploids.

Molecular and cytogenetic examination

Examined gynogenetic DH larvae showed only one of the alleles observed in the parental females, which proves that diploid gynogenetic DH larvae were homozygous individuals (Table 3). Analysis of the *sdY* marker confirmed that only parental male exhibited Y-chromosome-related DNA sequence (PCR product of approx. 400 bp size length). Neither parental female nor gynogenetic offspring exhibited Y-linked electrophoretic band. Brook trout from the control groups (non-HHP treated) and gynogenetic individuals had 84 chromosomes while specimens from triploid variant exhibited 126 chromosomes (Figure 2).

Table 3. Results of the microsatellite genotyping of gamete donors for the gynogenesis; brown trout (sperm donor) and brook trout (egg donor) and the brook trout from the mitogynogenetic group

Locus	Paternal genotype	Maternal genotype	Gynogenetic progeny genotype	The number of gynogenetic progeny
Sfo-12	na	200/280	200/200	5
			200/280	0
			280/280	5
Sfo-226	na	250/290	250/250	5
			250/290	0
			290/290	5
Sfo-D75	270/270	200/240	200/200	3
			200/240	0
			200/270	0
			240/240	7
			240/270	0

na – not amplified in brown trout.

Figure 2. Metaphase spreads of triploid ($3n=126$) (a) and diploid gynogenetic ($2n=84$) (b) brook trout specimens

Evaluation of malformation rate in groups

In both experiments, percentage of deformed larvae (collected from hatching to the swimming stage) in control groups reached 7.0%. In triploid groups (Experiment 1) the percentage of malformed individuals increased more than 2-fold in comparison to the control groups and equaled 19.1%. In Experiment 2, drastically increased incidences of deformed larvae (> 30%) were observed in both gynogenetic DH and meiogynogenetic groups (Figure 3).

Figure 3. Ratio of malformed brook trout individuals among Doubled Haploids (DH), meiogynogenotes (G) and their non-manipulated siblings (C)

Spinal deformities including kyphosis, scoliosis, lordosis and spiral specimens were observed in the larvae from all experimental groups. Most of the malformed individuals from the control groups showed scoliosis. Scoliosis and kyphosis were the most frequently observed malformations in fish from the triploid group. Most of the dead larvae from meiogynogenetic groups had scoliosis and kyphosis, while most of the dead DHs were c-shaped kyphotic larvae (Table 4, Figure 4). Overall the body curvature of malformed mitogynogenetic individuals surpassed the curvature of those meiogynogenetic. In several individuals more than one deformity was noticed (Table 4, Figure 4).

Table 4. Type and ratio of particular malformations in the control (C), meiogynogenetic (G), gynogenetic Doubled Haploids (DH) and triploid (3n) brook trout

	K	Sc	L	Sp	D	Ck	Cl	Si	K+Sc	L+Sc	H	H+Sc	H+L	H+K	H+Sc+L	H+Ck	H+Sc+K
C _{3n}	1.24	3.53	0.53	1.06	0.00	0.00	0.00	0.00	0.18	0.00	0.18	0.88	0.00	0.00	0.00	0.00	0.00
3n	4.32	8.47	1.26	0.54	0.18	0.18	0.00	0.00	1.80	0.18	0.36	0.72	0.18	0.54	0.36	0.00	0.00
C ₁	1.73	5.18	0.35	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.17	0.17	0.00	0.00	0.00	0.00
G ₁	5.75	20.31	0.77	0.00	0.38	1.15	0.00	0.00	0.77	0.77	0.77	0.77	0.77	1.15	0.00	0.00	0.00
DH ₁	6.15	3.08	0.00	0.77	0.00	14.62	3.85	0.77	0.00	0.77	0.77	0.00	0.00	1.54	0.00	2.31	0.77
C ₂	0.00	2.56	0.00	1.92	0.00	0.00	0.00	0.00	0.00	0.00	0.64	0.00	0.00	0.64	0.00	0.64	0.00
G ₂	0.00	25.00	0.00	0.00	0.00	0.00	0.00	0.00	12.50	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

Notes: K – kyphosis, Sc – scoliosis, L – lordosis, Sp – spiral larvae, D – double headed, Ck – c-shaped kyphotic, Cl – c-shaped lordotic, Sy – Siamese, H – head deformation (no eyes, swollen eyes, jaws deformation, shortened snout).

Figure 4. Examples of the body deformities among the chromosome set manipulated brook trout

Discussion

Triploid brook trout exhibited lower early survival rate than diploid specimens, however the differences were not significant. Reports on the survival of triploid fishes are inconsistent and several studies indicated decreased survivability of triploids in relation to diploids during early developmental stages while other experiments showed that juvenile diploid and triploid individuals had similar survival rates (Piferrer et al., 2009). Three sets of chromosomes give higher heterozygosity and potentially increased viability but on the other hand, during induction of triploidization, activated eggs are subjected to the sub-lethal environmental shock that has been suggested to reduce early survival of triploids (Cherfas et al., 1994). Contrary to triploid specimens, gynogenetic brook trout showed significantly lower survival than non-manipulated control fish and the Doubled Haploids exhibited substantially higher mortality when compared to the meiogynogenetic individuals (Figure 1). Taking into account that gynogenetic DH brook trout are fully homozygous individuals, the vast mortality of DHs might have resulted from expression of the recessive alleles. It has

been proved that manipulations applied for diploidization may also decrease developmental competence of the treated eggs (Komen and Thorgaard, 2007); thus, it is highly recommended to use only the best quality gametes for gynogenesis. In our study, eggs from one of the females showed reduced quality, which resulted in the low survival of its normal and gynogenetic progenies. In this particular case, none of the DHs survived till hatching (Figure 1). Low efficiency of mitotic gynogenesis caused that DHs have been produced only in a limited number of salmonid fish species including rainbow trout (Chourrout et al., 1984; Polonis et al., 2018) brown trout (*Salmo trutta*) (Michalik et al., 2015; Jagiełło et al., 2017) and amago salmon (*Oncorhynchus rhodurus*) (Kobayashi et al., 1994). Successful induction of brook trout DHs described in the present paper opens new opportunities for application of fully homozygous specimens in the breeding programs, production of clonal lines and whole genome sequencing that may be useful in aquaculture of *Salvelinus* fish.

Generally, genome manipulated and normal diploid brook trout studied in the present paper showed the same body disorders; however, triploids and gynogenotes had higher prevalence of deformations, which is in agreement with already published results (Fjelldal and Hansen, 2010; Fraser et al., 2013; Jagiełło et al., 2018, among others). The increased ratio of individuals with skeletal deformities among chromosome set manipulated brook trout may be caused by the low egg quality, side effects of the HHP shock, nutritional and genetic factors, and their interactions. Application of poor rainbow trout egg quality for gynogenesis resulted in high incidences of body malformations including emaciation and skeletal anomalies observed clonal individuals (Jagiełło et al., 2018). In turn, HHP shock applied for polyploidization has been found to impair embryonic development, including e.g. a delay of epiboly and suppression of the dorso-ventral differentiation (Yamaha et al., 2002). Triploid fish due to the altered gene expression and/or bigger cell size have different dietary and environmental requirements than diploids. Some studies show that triploid fish have increased requirement for the phosphorus and the presence of skeletal deformities may result from the insatiable demand for this mineral (Fjelldal et al., 2016).

Drastically increased ratio of specimens with body disorders was reported among brook trout from meio- and mitogynogenetic groups with scoliosis and kyphosis being the most frequently observed malformations (Table 4). Scoliosis was a spine curvature observed among normal brook trout from the control groups, and number of scoliotic individuals comprised as far as 25% of the meiogynogenetic specimens with the body deformations (Table 4). Interestingly, ratio of scoliosis in DH brook trout was lower than in their meiogynogenetic and non-manipulated (control) siblings. But this may be easily explained by the vast mortality of the DHs and presumed elimination of most of the deformed individuals during embryonic development. Incidences of kyphosis were also more frequently observed in gynogenotes than in their control siblings. Another issue that should be emphasized when analyzing body deformations in the brook trout examined here is a very high ratio of C-shaped larvae among DH specimens. As gynogenetic genome is of exclusive maternal origin and activated eggs are similarly treated (HHP shock) for gynogenesis and triploidization, higher incidences of skeletal deformities among diploid gynogenetic females including fully homozygous DHs suggested a genetic factor that

impaired early body development. It has been observed previously that increased homozygosity of fish is followed by the increased rate of larvae with vertebral deformities including lordosis, scoliosis and kyphosis (Aulstad et al., 1971; Shirak et al., 2012; Diekmann and Nagel, 2005; Jagiello et al., 2018). Nevertheless, the knowledge concerning the genetic background of the skeletal deformation in fish is still incomplete. Contribution of the genetic component to the spinal deformities has been also proposed in the grass carp (*Ctenopharyngodon idella*) (Grimmett et al., 2011). In zebrafish, one of the collagen types has been found to be crucial for the notochord morphogenesis and skeletogenesis. Knockdown of genes encoding collagen resulted in scoliosis (Christiansen et al., 2009). Another zebrafish gene that altered expression leads to scoliosis is the lady bird homebox (*lbx*) gene (Guo et al., 2016). Moreover, a QTL controlling susceptibility to the spinal curvature that act in a recessive manner has been described in guppy (*Poecilia reticulata*) (Gorman et al., 2011).

Conclusions

In the present research we compared survival and prevalence of spinal disorders in the meiogynogenetic, mitogynogenetic (DH) and triploid brook trout in relation to normal diploids. Although triploids and their diploid siblings show similar survivability, significantly increased mortality was observed among gynogenetic brook trout especially in the DH variant of the experiment. Body deformations were observed among fish from all groups, however intensification of kyphosis and scoliosis was clearly demonstrated in the gynogenetic and triploid individuals, which may be related to side effects of the egg treatment during polyploidization step and the genetic factors including increased number of chromosomes (triploids) and decreased heterozygosity (gynogenotes).

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Data Availability Statement

Data available on request due to privacy/ethical restrictions

Conflict of interest

None of the authors have any conflict of interest to declare.

Author contributions

KJ, KO and SD were involved in designing and performing the triploid and gynogenetic experiments. SD took care of the gynogenetic and control embryos and larvae. KJ and MP made molecular analysis. Identification and classification of body abnormalities among examined fishes were done by KJ. LP and KO prepared and analyzed chromosome spreads. KO and KJ were involved in data analysis, writing and editing the manuscript.

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