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Impact of selected physical and biological factors on efficiency of gynogenesis and androgenesis in rainbow trout (*Oncorhynchus mykiss*)

Wpływ wybranych czynników fizycznych i biologicznych na skuteczność gynogenezy i androgenezy pstrąga tęczowego (*Oncorhynchus mykiss*)

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Abstract

Introduction

At the end of the twentieth and in early twenty-first centuries many alarming reports on the poor status of fish stocks in the seas and oceans caused by overfishing were published. In the Mediterranean Sea alone, nearly 90% of fish stocks are below a stable level, which puts the entire ecosystem at the risk of imbalance. To meet the growing demand for fish and to protect overfished populations of economically important species of fish, aquaculture, i.e. the farming of aquatic organisms using techniques enhancing production beyond the natural capacity of the environment, has been strongly developed since the 1990s. The rapid growth of aquaculture has helped to master the production of many species of fish and aquatic invertebrates under controlled conditions. It has been estimated (FAO data) that by 2030, 65% of globally consumed aquatic organisms will come from aquaculture. The European leaders in aquaculture are Norway, the United Kingdom, Spain, France and Italy. Poland, with about 40,000 tonnes of fish (mainly salmonids and cyprinids) produced in aquaculture each year, is a significant supplier in the European Union. Rainbow trout (*Oncorhynchus mykiss*) is the most important species in Polish aquaculture and one of the most important in Europe, and its production in the EU accounts for approx. 240,000 tonnes, of which over 8% are trout from Poland.

Modern aquaculture owes its rapid growth not only to the increasing demand for fish and crustaceans, but also to new technologies allowing for the formulation of highly efficient fish feed, effective drugs and vaccines, and biotechnologies for the reproduction of fish under controlled conditions. Advances in genetics, molecular biology and biotechnology in recent decades also have their effect on today's aquaculture. For example, methods of reproductive biotechnology, such as androgenesis and gynogenesis, have been used for several years in basic and applied studies related to ichthyology and aquaculture (Komen and Thorgaard, 2007). In these techniques, gametes are inactivated by exposure to high doses of ionizing or UV radiation, which destroy nuclear DNA. Androgenetic haploid embryos are obtained by the insemination of inactivated eggs with normal sperm, while gynogenetic haploid embryos are produced by the activation of eggs using sperm

with destroyed nuclear DNA. In the next stage, the haploid zygotes are exposed to high hydrostatic pressure or sublethal temperature to inhibit the first division of the nucleus, duplicate the paternal (androgenesis) or maternal (mitotic gynogenesis) chromosomes and produce doubled haploids (DHs), which are fully homozygous (Pandian and Koteeswaran, 1998). There is also the second variant of gynogenesis called meiotic gynogenesis. In this process the haploid zygotes are exposed to the environmental shock a little earlier, shortly after insemination. As a result, the production of the second polar body is arrested, the diploid number of chromosomes is restored, and the individuals obtained in this way are heterozygous (Komen and Thorgaard, 2007).

Haploid androgenetic and gynogenetic embryos and doubled haploids are used in studies investigating the function of individual genes and the influence of recessive alleles on ontogenesis (Zhang et al., 2014). Moreover, the genomes of homozygous doubled haploids are more easily sequenced (Felip et al., 2001). Because only maternal or paternal chromosomes are inherited, androgenesis and gynogenesis are useful in research on genetic sex determination in fish (Komen and Thorgaard, 2007). Androgenesis and gynogenesis are used in aquaculture selection programmes for the production of isogenic fish stocks or stocks composed of single-sex fish, as well as clonal lines (Hulata, 2001; Billard, 1992). Additionally, androgenesis helps protect and restore gene pools of populations, lines and even species of fish (interspecies androgenesis) from sperm stored in liquid nitrogen (Babiak et al., 2002). Androgenesis and gynogenesis are usually induced in fish species (sometimes in aquatic invertebrates) important for aquaculture, i.e., mainly salmonids (Salmonidae) (Salmonidae) (Chourrout, 1984), cyprinids (Cyprinidae) (Komen et al., 1991), European sea bream (*Sparus aurata*) (Peruzzi and Chatain, 2000), sea bass (*Dicentrarchus labrax*) (Francescon et al., 2004), turbot (*Scophthalmus maximus*) (Piferrer et al., 2004), and halibut (*Hippoglossus hippoglossus*) (Tvedt, 2006). Unfortunately, there are some limitations preventing the wider use of both techniques, for example the relatively low survival of androgenetic and gynogenetic offspring, which is associated with the expression of lethal alleles (Komen and Thorgaard 2007), but probably also with a low quality of the female gametes used in these studies. Considering the fact that fish eggs during induced androgenesis and gynogenesis are exposed to harmful physical factors, the roe used

in these procedures should be of the highest quality, i.e., characterized primarily by normal morphology, high post-fertilization survival and normal development of embryos (Aegerter and Jalabert, 2004; Migaud et al., 2013). The quality of female gametes in fish depends on many factors, including the health of the spawners or the environmental conditions in which the fish live before spawning (water quality, food quality, farming conditions, exposure to stress). Post-ovulatory ageing of eggs inside the fish body or hormonal stimulation used to induce ovulation, and the stress caused by the breeding conditions significantly reduce fertilization capacity and have a negative effect on the development of embryos (Aegerter and Jalabert, 2004; Migaud et al., 2013). It was recently confirmed that the developmental competence of eggs is also influenced by the quality of the maternal RNA accumulated in oocytes during oogenesis, and this quality depends on the combined effect of environmental factors to which the spawners and female genome are exposed (Aegerter et al., 2005) (Figure 1).

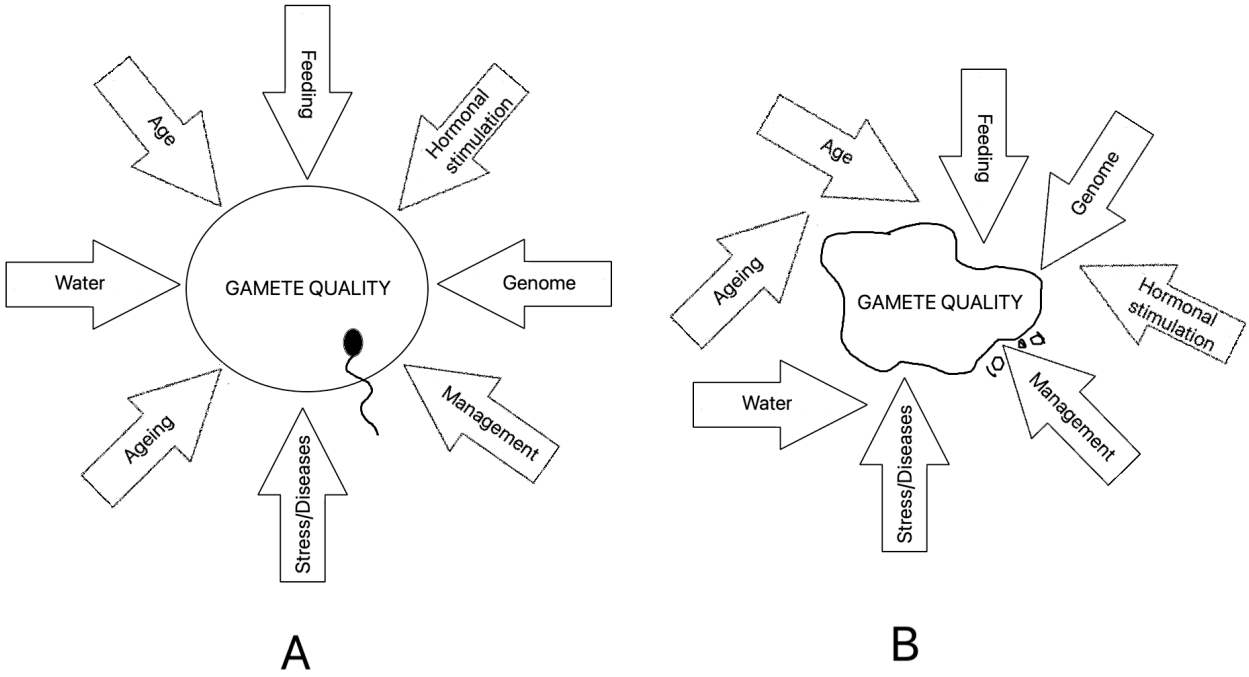

Figure 1. Factors that determine gamete quality in fish. A - balanced; B - unbalanced.

The early stages of embryonic development in fish are completely determined by maternal mRNA, which is deposited in oocytes during oogenesis and regulates a series of synchronous cell divisions prior to the activation of the zygotic genome (Kane and Kimmel, 1993; Sullivan et al., 2015). Studies carried out by dr hab. Konrad

Ocalewicz and collaborators on the maternal transcriptome using next-generation sequencing revealed significant differences in the expression of several dozen genes in eggs from different female rainbow trout (Gurgul et al., 2018). Therefore, it was assumed that gametes from different females might have different transcriptomic profiles and different developmental competence after gynogenetic activation. In selected fish species, such as European sea trout (*Salmo trutta fario*) or Arctic char (*Salvelinus alpinus*), high egg quality may be manifested by the even distribution of lipid droplets, while the accumulation of droplets on one of the poles of the egg indicates that these cells have limited developmental competence (Mansour et al., 2007; Mansour et al., 2008; Ciereszko et al., 2009). The survival rate of embryos developing in such eggs is usually much lower, and the percentage of malformed individuals that hatch from them is higher (Aegerter and Jalabert, 2004).

Bearing in mind that environmental factors have a critical impact on the quality of fish eggs, it should be presumed that both the irradiation of gametes and the exposure of fertilized eggs to high hydrostatic pressure may have a negative effect on the survival of the embryos developing inside eggs treated in this way. The use of insufficiently high doses of UV light for the irradiation of sperm during gynogenesis leads to incomplete inactivation of nuclear DNA in spermatozoa. For this reason, fragments of paternal chromosomes might still be present in the cells of gynogenetic individuals. Chromosome fragments may destabilize the gynogenetic genome and thus reduce the survival of carriers of paternal chromosome fragments (Chourrout, 1984, Michalik et al., 2015). One mechanism preventing interspecies hybridization relies on the removal of only paternal chromosomes from the cells of the hybrid embryos. Therefore, the use of gametes from different species to induce gynogenesis may solve the problem of contamination with fragments of irradiated chromosomes. It is also possible that the use of ionizing radiation to irradiate female gametes during androgenesis and the exposure of haploid embryos to high pressure shock for diploidization may in turn destabilize the cytoskeleton and impair the function of microtubules responsible, among others, for the transport of lipid droplets in the cytoplasm, and cause destruction of maternal RNA (Parker et al., 2014; Aegerter et al., 2005). Additionally, ionizing radiation used for the inactivation of nuclear DNA in eggs during androgenesis is one of the main sources of reactive oxygen species (ROS) (Samarin et al., 2018), and their excessive level may reduce

the quality of fish eggs and the survival rate of embryos developing inside them. The activity of enzymatic antioxidant system, including superoxide dismutase (SOD), catalase (CAT) or glutathione peroxidase (GPx), might also increase inside the cells in response to the increased ROS levels.

Research motivation and summary of the results

The general objective of this study was to analyse the influence of selected physical and biological factors on the efficiency of gynogenesis and androgenesis in rainbow trout (*Oncorhynchus mykiss*), while the specific objectives included the production of gynogenetic rainbow trout using eggs activated with UV-irradiated spermatozoa from brown trout (*Salmo trutta*) [Publication 1], analysis of the distribution of lipid droplets in the cytoplasm of eggs exposed to ionizing radiation during induced androgenesis and assessment of their developmental competence [Publication 2], identification of candidate genes influencing the efficiency of induced gynogenesis [Publication 3], and evaluation of the activity of antioxidant enzymes in eggs exposed to ionizing radiation during androgenesis [Publication 4].

The experiments aimed at achieving these research objectives were carried out in 2015-2019 based on the approval from the Local Ethical Committee for Animal Experiments in Gdańsk (no. 28/2015). The gametes used in the research were acquired from rainbow trout (*Oncorhynchus mykiss*) and brown trout (*Salmo trutta*) farmed at the Department of Salmonid Research (ZHRŁ) in Rutki (Inland Fisheries Institute in Olsztyn). Roe used in studies on androgenesis was transported to the Department of Oncology and Radiotherapy, Clinical Centre of the Medical University of Gdańsk, where it was irradiated with X-rays using the TrueBeam linear accelerator. The irradiated roe was transported to the institute in Rutki to carry out further stages of experiments. Irradiated and non-manipulated roe was fertilized to produce haploid androgenotes and diploids whose genome included the chromosomes of both parents (control groups). In gynogenesis, non-manipulated eggs were activated by spermatozoa previously irradiated with UV to inactivate the paternal chromosomes. Androgenetic and gynogenetic haploid zygotes were exposed to high hydrostatic pressure in order to restore the diploidy in embryos (Michalik et al., 2015). During the presented doctoral studies, experiments on gynogenesis and androgenesis were carried out twice. The survival rates of

gynogenotes, androgenotes, control fish, the percentage of malformed larvae, as well as the type of deformities (analysed during the early stages of ontogenesis) were the basic parameters describing the quality of eggs used in the research and the efficiency of genomic manipulations. In addition, eggs were analysed for the distribution of lipid droplets, the pH of the ovarian fluid, the activity of antioxidant enzymes, and the quantity and quality of the maternal transcriptome. The homozygosity of androgenetic and gynogenetic individuals was confirmed by microsatellite DNA analysis (Kaczmarczyk and Kaczor, 2013; Rexroad et al., 2002; Rexroad et al., 2001; Rexroad and Palti, 2003). The effectiveness of nuclear DNA inactivation and cell ploidy were analysed using cytogenetic diagnostic techniques (Ocalewicz et al., 2013). The motility of the activated and irradiated spermatozoa used in the subsequent experiments was examined under a microscope.

Despite the very high quality of gametes used in individual experiments, the survival of androgenetic and gynogenetic embryos and hatchlings was significantly lower than in the control groups [Publications 1,2,3,4]. Moreover, the analysis of larval development showed a significantly higher incidence of anatomical anomalies in homozygous androgenetic doubled haploids compared to heterozygous control fish [Publication 2].

Rainbow trout and brown trout hybrids were non-viable because of large interspecies differences in genome structure and organization. For this reason, sperm from rainbow trout and brown trout was used in a comparative study to induce gynogenesis in rainbow trout. Cytogenetic analysis of hybrid embryos showed inter-individual and intra-individual differences in the number of chromosomes and the presence of chromosome fragments. The survival of rainbow trout gynogenetic embryos and larvae was slightly higher in the groups where homologous sperm was used ($p > 0.05$). No chromosome fragments were found in the gynogenetic cells of rainbow trout that hatched from eggs activated with irradiated homologous and heterologous sperm [Publication 1].

Eggs from four females before and after exposure to X-rays during induced androgenesis were compared to estimate the effect of ionizing radiation on the distribution of lipid droplets. The distribution of the lipid droplets in most of the analyzed eggs before irradiation was relatively even. In the groups of eggs that were exposed to irradiation, an increased number of gametes characterized by uneven

distribution of lipid droplets was observed. Moreover, the incidence of malformations was higher in fish embryos developing in eggs exposed to ionizing radiation before fertilization. Only a few androgenotes survived to the swim-up stage [Publication 2].

Another experiment revealed significant differences in the survival of gynogenetic embryos developing in eggs from different females. Eggs from one of the four tested females showed a special competence for gynogenetic development. Embryos developing in these eggs were characterized by an almost 10-fold higher survival rate compared to embryos developing in eggs collected from other females. Comparative analysis of egg transcripts with different developmental competences after activation with irradiated sperm identified 46 genes whose expression was significantly higher in eggs characterized by high survival of gynogenetic trout developing inside them. Functional analysis of the transcripts of these genes showed their involvement, for example, in processes related to cell differentiation, early embryonic development, triglyceride metabolism, biosynthesis of polyunsaturated fatty acids, as well as cell ageing [Publication 3].

Ionizing radiation is one of the main factors causing cell damage and stress by disrupting DNA integrity and the formation of ROS. Therefore, when planning the last experiment, it was assumed that the activity of antioxidant enzymes in eggs exposed to ionizing radiation during androgenesis may change. It was also assumed that the pH of the ovarian fluid would decrease after irradiation. Survival, the activity of antioxidant enzymes, including SOD, CAT and GPx, as well as the pH of the ovarian fluid were tested in non-irradiated and irradiated eggs from four females. The survival of androgenetic embryos developing in eggs from different females varied considerably, and the differences were significant. Significant differences in the activity of antioxidant enzymes were also found for eggs from different rainbow trout females. The eggs from the female which showed the highest developmental competences for androgenesis were also characterized by increased activity of SOD, CAT and GPx. In most cases, the study also confirmed changes in enzymatic activity between unexposed and irradiated eggs from the same female. The pH of the ovarian fluid for each of the females was over 8 before and after irradiation [Publication 4].

The studies presented in this doctoral dissertation demonstrated that UV-inactivated sperm from brown trout can be successfully used to induce gynogenesis in rainbow trout eggs [Publication 1]. Changes observed in irradiated eggs indicate that exposure of rainbow trout eggs to ionizing radiation during androgenesis increases the number of gametes with unevenly distributed lipid droplets. Moreover, androgenetic embryos of rainbow trout developing in irradiated eggs rarely hatched, and a large part of them had multiple malformations due to the expression of recessive alleles, but also indicating reduced developmental competences of irradiated female gametes [Publication 2]. The genes that showed overexpression in eggs for which clearly higher survival of gynogenetic rainbow trout was observed can be considered candidate genes for the efficiency of the induced gynogenesis. Among them, genes related to early embryonic development, lipid metabolism and cell ageing deserve special attention [Publication 3]. Considerable differences in the activity of antioxidant enzymes and significant differences in the survival of androgenotes developing in eggs from different females suggest that maternal genetic factors have a much greater influence on the quality of eggs and the normal development of androgenotes than ionizing radiation alone. Moreover, the influence of ionizing radiation on the pH of ovarian fluid, which was originally considered important for the survival of androgenetic trout, should be disregarded [Publication 4].

Bibliography

Aegerter, S., & Jalabert, B. (2004). Effects of post-ovulatory oocyte ageing and temperature on egg quality and on the occurrence of triploid fry in rainbow trout, *Oncorhynchus mykiss*. *Aquaculture*, *231*(1-4), 59-71. https://doi.org/10.1016/j.aquaculture.2003.08.019

Aegerter, S., Jalabert, B., & Bobe, J. (2005). Large scale real-time PCR analysis of mRNA abundance in rainbow trout eggs in relationship with egg quality and post-ovulatory ageing. *Molecular Reproduction and Development: Incorporating Gamete Research*, *72*(3), 377-385. https://doi.org/10.1002/mrd.20361

Babiak, I., Dobosz, S., Goryczko, K., Kuzminski, H., Brzuzan, P., & Ciesielski, S. (2002). Androgenesis in rainbow trout using cryopreserved spermatozoa: the effect of processing and biological factors. *Theriogenology*, *57*(4), 1229-1249. https://doi.org/10.1016/S0093-691X(02)00631-3

Billard, R. (1992). Reproduction in rainbow trout: sex differentiation, dynamics of gametogenesis, biology and preservation of gametes. *Aquaculture*, *100*(1-3), 263-298. https://doi.org/10.1016/0044-8486(92)90385-X

Chourrout, D. (1984). Pressure-induced retention of second polar body and suppression of first cleavage in rainbow trout: production of all-triploids, all-tetraploids, and heterozygous and homozygous diploid gynogenetics. *Aquaculture*, *36*(1-2), 111-126. https://doi.org/10.1016/0044-8486(84)90058-9

Ciereszko, A., Wojtczak, M., Dietrich, G. J., Kuźmiński, H., & Dobosz, S. (2009). A lack of consistent relationship between distribution of lipid droplets and egg quality in hatchery-raised rainbow trout, Oncorhynchus mykiss. *Aquaculture*, *289*(1-2), 150-153. https://doi.org/10.1016/j.aquaculture.2008.12.032

Felip, A., Zanuy, S., Carrillo, M., & Piferrer, F. (2001). Induction of triploidy and gynogenesis in teleost fish with emphasis on marine

species. *Genetica*, *111*(1), 175-195. https://doi.org/10.1023/A:1013724322169

Francescon, A., Libertini, A., Bertotto, D., & Barbaro, A. (2004). Shock timing in mitogynogenesis and tetraploidization of the European sea bass *Dicentrarchus labrax*. *Aquaculture*, *236*(1-4),201-209. https://doi.org/10.1016/j.aquaculture.2003.10.018

Gurgul, A., Pawlina-Tyszko, K., Bugno-Poniewierska, M., Szmatoła, T., Jasielczuk, I., Dobosz, S., & Ocalewicz, K. (2018). Transcriptome analysis of rainbow trout (*Oncorhynchus mykiss*) eggs subjected to the high hydrostatic pressure treatment. *International Journal of Genomics*, *2018,* 1–7. https://doi.org/10.1155/2018/5197126

Hulata, G. (2001). Genetic manipulations in aquaculture: a review of stock improvement by classical and modern technologies. *Genetica*, *111*(1), 155-173. https://doi.org/10.1023/A:1013776931796

Kaczmarczyk, D., & Kaczor, A. (2013). New multiplex PCR assays for estimating genetic diversity in rainbow trout (*Oncorhynchus mykiss*) by polymorphism of microsatellite DNA. *Environmental Biotechnology*, *9,* 19–24.

Kane, D. A., & Kimmel, C. B. (1993). The zebrafish midblastula transition. *Development*, *119*(2), 447-456. https://doi.org/10.1242/dev.119.2.447

Komen, H., & Thorgaard, G. H. (2007). Androgenesis, gynogenesis and the production of clones in fishes: a review. *Aquaculture*, *269*(1-4), 150-173. https://doi.org/10.1016/j.aquaculture.2007.05.009

Komen, J., Bongers, A. B. J., Richter, C. J. J., Van Muiswinkel, W. B., & Huisman, E. A. (1991). Gynogenesis in common carp (Cyprinus carpio L.): II. The production of homozygous gynogenetic clones and F1 hybrids. *Aquaculture*, *92*, 127-142. https://doi.org/10.1016/0044-8486(91)90015-YMansour, N., Lahnsteiner, F., & Patzner, R. A. (2007). Distribution of lipid droplets is an indicator for egg quality in brown trout, *Salmo trutta fario*. *Aquaculture*, *273*(4), 744-747. https://doi.org/10.1016/j.aquaculture.2007.09.027

Mansour, N., Lahnsteiner, F., McNiven, M. A., & Richardson, G. F. (2008). Morphological characterization of Arctic char, *Salvelinus alpinus*, eggs

subjected to rapid post-ovulatory aging at 7 C. *Aquaculture*, *279*(1-4), 204-208. https://doi.org/10.1016/j.aquaculture.2008.04.014

Michalik, O., Dobosz, S., Zalewski, T., Sapota, M., & Ocalewicz, K. (2015). Induction of gynogenetic and androgenetic haploid and doubled haploid development in the brown trout (*Salmo trutta,* Linnaeus 1758). *Reproduction in Domestic Animals*, *50*(2), 256-262. https://doi.org/10.1111/rda.12480

Migaud, H., Bell, G., Cabrita, E., McAndrew, B., Davie, A. et al. (2013). Gamete quality and broodstock management in temperate fish. *Reviews in Aquaculture*, 5, 194–223. https://doi.org/10.1111/raq.12025

Ocalewicz, K., Kuzminski, H., Pomianowski, K., & Dobosz, S. (2013). Induction of androgenetic development of the brook charr (*Salvelinus fontinalis*) × Arctic charr (*Salvelinus alpinus*) hybrids in eggs derived from the parental species. *Reproductive Biology*, *13*(2), 105-112. https://doi.org/10.1016/j.repbio.2013.03.002

Pandian, T. A., & Koteeswaran, R. (1998). Ploidy induction and sex control in fish. *Hydrobiologia*, *384*(1), 167-243. https://doi.org/10.1023/A:1003332526659

Parker, A. L., Kavallaris, M., & McCarroll, J. A. (2014). Microtubules and their role in cellular stress in cancer. *Frontiers in Oncology*, *4*, 153. https://doi.org/10.3389/fonc.2014.00153

Peruzzi, S., & Chatain, B. (2000). Pressure and cold shock induction of meiotic gynogenesis and triploidy in the European sea bass, *Dicentrarchus labrax L.*: relative efficiency of methods and parental variability. *Aquaculture*, *189*(1-2), 23-37. https://doi.org/10.1016/S0044-8486(00)00355-0

Piferrer, F., Cal, R. M., Gómez, C., Álvarez-Blázquez, B., Castro, J., & Martínez, P. (2004). Induction of gynogenesis in the turbot (*Scophthalmus maximus*): effects of UV irradiation on sperm motility, the Hertwig effect and viability during the first 6 months of age. *Aquaculture*, *238*(1-4), 403-419. https://doi.org/10.1016/j.aquaculture.2004.05.009

Rexroad 3rd, C. E., Coleman, R. L., Martin, A. M., Hershberger, W. K., & Killefer, J. (2001). Thirty-five polymorphic microsatellite markers for rainbow trout

(*Oncorhynchus mykiss*). *Animal Genetics*, *32*(5), 317-319. https://doi.org/10.1046/j.1365-2052.2001. 0730b.x

Rexroad III, C. E., & Palti, Y. (2003). Development of ninety-seven polymorphic microsatellite markers for rainbow trout. *Transactions of the American Fisheries Society*, *132*(6), 1214-1221. https://doi. org/10.1577/T02-086

Rexroad Iii, C. E., Coleman, R. L., Gustafson, A. L., Hershberger, W. K., & Killefer, J. (2002). Development of rainbow trout microsatellite markers from repeat enriched libraries. *Marine Biotechnology*, *4*(1), 12-16. https://doi.org/10.1007/s10126-001-0058-6

Samarin, A. M., Samarin, A. M., & Policar, T. (2019). Cellular and molecular changes associated with fish oocyte ageing. *Reviews in Aquaculture*, *11*(3), 619-630. https://doi.org/10.1111/raq.12249

Sullivan, C. V., Chapman, R. W., Reading, B. J., & Anderson, P. E. (2015). Transcriptomics of mRNA and egg quality in farmed fish: some recent developments and future directions. *General and comparative endocrinology*, *221*, 23-30. https://doi.org/10.1016/j.ygcen.2015.02.012

Tvedt, H. B., Benfey, T. J., Martin-Robichaud, D. J., McGowan, C., & Reith, M. (2006). Gynogenesis and sex determination in Atlantic halibut (*Hippoglossus hippoglossus*). *Aquaculture*, *252*(2-4), 573-583. https://doi.org/10.1016/j.aquaculture.2005.06.042

Zhang H, Tan E, Suzuki Y, Hirose Y, Kinoshita S, Okano H, Kudoh J, Shimizu A, Saito K, Watabe S, Asakawa S (2014). Dramatic im- provement in genome assembly achieved using doubled-haploid genomes. *Scientific Reports*, 4(1), 1-5. https://doi.org/10.1038/srep06780