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ROZPRAWA DOKTORSKA

Changes in the length of telomeric DNA and telomerase activity in diploid and triploid rainbow trout (*Oncorhynchus mykiss*)

Zmiany długości telomerowego DNA i aktywności telomerazy u diploidalnych i triploidalnych pstrągów tęczowych (*Oncorhynchus mykiss*)

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List of publications constituting the doctoral thesis

Publication1: Panasiak, L., Dobosz, S., i Ocalewicz, K. (2020). Telomere dynamics in the diploid and triploid rainbow trout (*Oncorhynchus mykiss*) assessed by Q-FISH analysis. *Genes*, 11(7), 786. https://doi.org/10.3390/genes11070786

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Publication4: Panasiak, L., Kuciński, M., Hliwa, P., Pomianowski, K. i Ocalewicz, K. (2023). Telomerase activity in somatic tissues and ovaries of diploid and triploid rainbow trout (*Oncorhynchus mykiss*) females. *Cells*, 2446865.

Abstract

Telomeres are conserved regions of the genome located at the ends of eukaryotic chromosomes, consisting of short and repetitive DNA sequences (5'-TTAGGG-3') and proteins (shelterin). Telomeres shorten during each cell division cycle, which is associated with the ageing process. The rate of telomeric DNA shortening is an individual characteristic influenced by genetic and environmental factors. Telomerase is a ribonucleoprotein enzyme whose task is to synthesise DNA strands in the telomere area, which slows down the process of its shortening. Telomerase consists of two key components: the catalytic *Tert* subunit with the nature of telomerase reverse transcriptase, and the TERC subunit, which serves as the internal RNA template. In most warm-blooded organisms, telomerase is not active in somatic cells. On the other hand, in cold-blooded organisms such as fish, telomerase activity is observed in somatic cells throughout their lifetime. For this reason, telomeric DNA in fish does not always shorten with age, and the dynamics of changes in telomere length may look different in various species. In addition, it is assumed that the constant expression of telomerase in fish is associated with, among other things, excellent tissue regenerative capacity and increased resistance to cancer. Triploid individuals are an excellent model for research on the dynamics of changes in telomeric DNA length and telomerase activity because of their unique genetic and physiological characteristics; larger cell size, higher heterozygosity, inhibited development of gonads and limited production of gametes, continuous growth, as well as reduced resistance to adverse environmental conditions.

In the present study, changes in the length of telomeric DNA, as well as the expression of the *Tert* gene and the activity of the telomerase enzyme in selected somatic tissues and in the gonads of diploid (2n) and triploid (3n) rainbow trouts (*Oncorhynchus mykiss*) at different ages and in individuals characterised with different growth rates, were investigated. The average length of the $(TTAGGG)_n$ sequence of one-year-old rainbow trouts was 20,000 base pairs. Differences in telomere length between males and females were not significant. The study has shown that the dynamics of changes in telomeric DNA length in diploid and triploid fish was similar, which suggests that the additional set of chromosomes in triploid fish and all its consequences have a limited impact on telomere length in this species. In the case of individuals with dwarfism and in those with normal development, no significant differences in telomere length were found. The activity of telomerase in selected tissues of normally developing individuals and dwarf individuals also did not differ significantly, except that it

was lower in the skin in fish with dwarfism. An increased level of expression of the *Tert* gene was found in the liver, spleen, muscles and gills of triploid individuals, which seems to be of great importance in maintaining cell homoeostasis in individuals that, compared to diploid fish, are definitely more environmentally demanding. However, in the ovaries of triploid fish, expression of the *Tert* gene was significantly lower compared to the gonads of diploid females. The ovaries of triploid rainbow trouts were strongly reduced and contained few oocytes. The small number of reproductive cells, which are usually characterised by high telomerase activity, probably contributed to the low expression of the *Tert* gene observed in sterile ovaries.

Key words: telomeric DNA, telomerase, rainbow trout, triploidisation, growth deficiency

Introduction

Telomeres and telomerase

Telomeres are non-coding regions of the genome consisting of tandemly repeated TTAGGG sequences, which together with a complex of protective proteins (shelterins – POT1, TPP1, TRF1, TRF2, RAP1, TIN2) are located at the ends of eukaryotic chromosomes (Figure 1) [1]. Telomeres stabilise the structure of chromosomes and protect their internal regions against damage to the contained genetic information during cell divisions. In addition, telomeres regulate the expression of genes located in the vicinity of the telomeric region, enable repair systems to recognise both normal and damaged chromosome ends, prevent chromosomal mutations (translocations, duplications, deletions) and ensure the correct course of the recombination process as well as enable the spatial organisation of the cell nucleus [2-5].

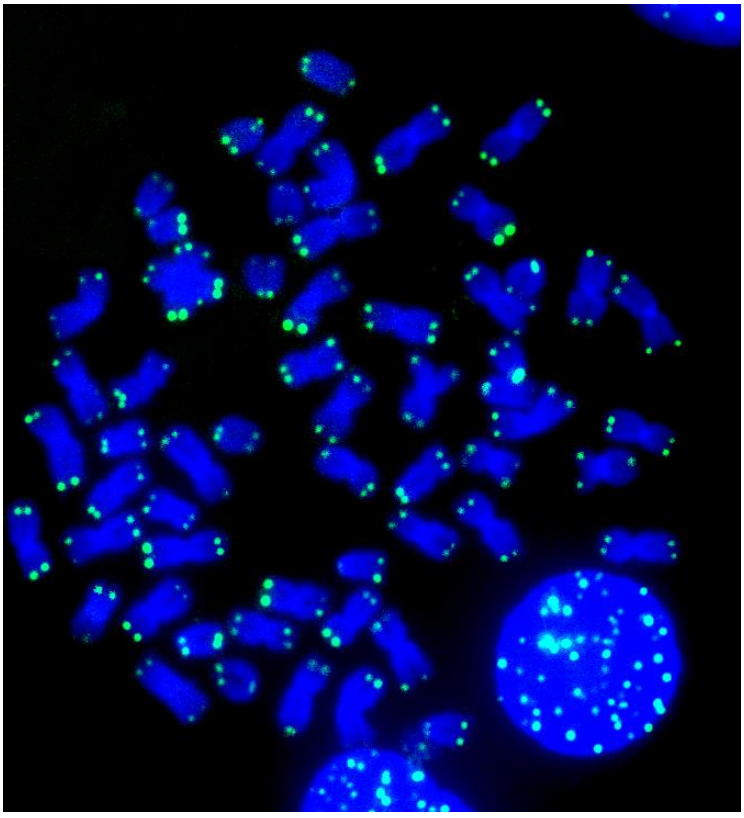


Figure 1 *Rainbow trout chromosomes hybridized with a PNA telomeric probe labeled with fluorescein isothiocyanate (FITC).*

In humans, as well as in the vast majority of mammals, telomeres shorten with age, which is a natural consequence of cell division [6, 7]. Linear eukaryotic chromosomes shorten during replication, and this phenomenon is known as the 'end replication problem'. Since DNA polymerase requires an RNA primer to initiate synthesis in the 5' → 3' direction, only the DNA leading strand can be synthesised to the very end. In the case of the lagging strand, which is synthesised from many small DNA fragments, called Okazaki fragments, it cannot be fully synthesised because there is no way to synthesise the last Okazaki fragment, as the primer would have to be located beyond the end of the chromosome. Otherwise, the attached primer of the last Okazaki fragment cannot be replaced by DNA polymerase as is done in the case of the others. As a result, part of the sequence located at the end of the replicated DNA remains uncopied, creating a single-stranded structure called *overhang* [8]. As a result of this process, with each cell cycle, telomeres are gradually shortened at a rate of 50 to 200 nucleotides [9]. This continues until they reach a critical length, which is the message to stop cell division and start the process of programmed cell death (apoptosis). The shortening of telomeric DNA is associated with the ageing process, as well as with the development of, among other diseases, cancer, cardiovascular diseases, diabetes, neurodegenerative diseases (Alzheimer's disease) or genetic disorders (Werner syndrome, Bloom syndrome) [10–12]. The progressive loss of the telomeric DNA sequences in human somatic cells is considered a mechanism of tumour suppression, while some cells have a faulty response to DNA damage and continue to grow despite the presence of telomere dysfunction [13]. The results of numerous studies indicate that oxidative stress is an important factor accelerating the rate of telomere shortening [14, 15]. Oxidative stress is defined as the disproportion between the rate of production of reactive oxygen species (ROS), and their neutralisation by antioxidant systems [16]. Telomeric DNA is rich in guanine nucleotides, which are particularly susceptible to damage caused by reactive oxygen species [14]. In the event that cellular homoeostasis is disturbed, which is caused by excessive production of ROS and impaired response mechanisms to oxidative stress, the rate of telomere shortening may increase, which also translates into the rate of ageing of the organism [15]. Reactive oxygen species are produced during metabolic processes, but their level may increase as a result of exposure to factors such as a highly processed diet low in antioxidants, stress, UV radiation or environmental pollution [15, 17, 18]. All of these factors have a negative impact on maintaining the appropriate length of telomeres. The average length of telomeres is species-specific, but differences in telomere length and rate of shortening have also been observed between individuals of the same species. In addition, the length of telomeric DNA can be

different in the cells of individual tissues/organs of the same organism. For example, in humans, the length of the $(TTAGGG)_n$ sequence ranges from approximately 5,000 to 15,000 nucleotides [19]. It has been noticed that the length of telomeric DNA varies depending on sex, and that women usually have longer telomeres than men [20, 21]. This may be a result of the fact than women are characterised by higher levels of oestrogens, which has anti-inflammatory and antioxidant effects.

Studies concerning the chromosomal location of the telomeric sequence have been performed in approximately 80 species of fish. The length of telomeric DNA in fish ranges from approximately 3,000 base pairs (common torpedo (*Torpedo ocellata*)) to even 25,000 base pairs (zebra fish (*Danio rerio*)) [22, 23]. The telomeres in fish, similarly to humans, can shorten with age, which has been observed in the turquoise killifish (*Nothobranchius furzeri*), the Siberian sturgeon (*Acipenser baeri*) and the western mosquitofish (*Gambusia affinis*) [24–26]. On the other hand, telomere length of the black buffalo (*Ictiobus cyprinellus*) does not change throughout its lifespan [27]. It is yet different in zebra fish, in whose cells the telomeric DNA lengthens and shortens depending on the stage of development at which the fish is at a given moment [28]. Studies conducted on the ninespine stickleback (*Pungitius pungitius*) have shown that in females of this species, telomere shortening progresses with the achievement of sexual maturity, which suggests that sexual maturation processes requiring more energy in females may lead to oxidative stress and, consequently, accelerate shortening of telomeric DNA. Such changes have not been observed in males of this species [29].

Telomerase is a ribonucleoprotein enzyme that plays a key role in maintaining telomere length and integrity. This enzyme is responsible for the synthesis of telomeric DNA sequences, slowing down or preventing telomere shortening. Telomerase consists of the *Tert* catalytic subunit with the nature of telomerase reverse transcriptase, the TERC subunit consisting of an RNA molecule that serves as a template for the synthesis of telomeric DNA, and the dyskerin protein necessary to maintain the spatial structure of telomeres [1, 30]. In addition to maintaining the appropriate length of telomeres, telomerase plays a significant role, among other things, in the regeneration of damaged tissues, in the process of carcinogenesis, antioxidant protection and in the ageing mechanism of the body [31, 32]. In most warm-blooded organisms, telomerase activity has been confirmed only in germline cells, stem cells and cancer cells [31]. However, research shows that telomerase expression can be induced during tissue regeneration or wound healing [33]. Interestingly, mice with the *Tert*

gene turned off lose the ability to regenerate tissues, including skin, which is manifested by hair loss and greying, as well as reduced wound healing capacity [34, 35]. In contrast to mammals, in which telomerase activity is largely limited, fish telomerase is active in cells of all tissues, regardless of the age of the examined individuals, which has been described in several species [23, 24, 28, 36–40]. Studies on model fish species show a high correlation between the expression of the *Tert* gene and the activity of telomerase, which suggests that the regulation of transcription of this gene is one of the basic mechanisms regulating the activity of the enzyme [40]. The first fish species in which such widespread telomerase activity was confirmed, also in older individuals, was the rainbow trout [36]. In telomerase-deficient zebra fish *(Tert*$^{-/-}$*)*, premature infertility, tissue atrophy, weight loss and exacerbation of inflammation were observed [41]. The turquoise killifish with the *Tert* gene turned off using the CRISPR/Cas9 technique were characterised by reduced fertility and the presence of atrophic testes and ovaries [42]. It seems, therefore, that the high activity of telomerase in fish, in addition to the obvious functions related to the control of telomere length, also ensures the maintenance of homoeostasis of organs and tissues.

The relationship between telomere length and telomerase activity, and fish body weight is a complex issue. The length of telomeric DNA is a genetically determined feature, but the dynamics of changes in the length of the sequence $(TTAGGG)_n$ is the function of growth rate, number of cell divisions, exposure to oxidative stress and telomerase activity, which in the case of cold-blooded organisms is present in somatic cells [43]. The basic function of telomerase is the addition of nucleotides to the telomeric sequence in each cycle of cell division, which may explain the lack of correlation between telomere length and age in the common garter snake (*Thamnophis sirtalis*), the leatherback sea turtle (*Dermochelys coriacea*) [44, 45] or several species of fish, where telomere length in adult individuals is comparable to that observed in juvenile fish [27]. But this is not the rule. In the case of fish, the rapid growth rate from hatching to sexual maturity may lead to telomere shortening, which is observed in the cells of several species studied in this respect [37, 46]. Fast-growing transgenic Pacific salmon (*Oncorhynchus kisutch*) with extra copies of the growth hormone gene are characterised by shorter telomeres compared to non-transgenic control fish. Moreover, during the period of rapid growth, the rate of shortening of telomeric DNA in transgenic individuals was significantly faster [47]. The increased production of free radicals accompanying the intensive growth of fish can cause oxidative stress, which in turn contributes to the increase in the rate of the ‘erosion’ of telomeres. Therefore, it seems, that

telomerase is not always able to compensate for the loss of telomeric DNA resulting from intensive cell proliferation during rapid somatic growth. It is also difficult to look for a single model of the relationship between telomere length and body size or weight in cold-blooded animals. In the American alligator (*Alligator mississippiensis*), individuals with a longer body length had shorter telomeres [48]. In turn, in the Eurasian carp, the length of telomeric DNA increased with the length of the fish body [49]. Telomerase activity in muscle cells may be relatively low in adult fish, which inevitably have a higher body weight than juveniles, as is observed in rainbow trout and cod [36, 39]. On the other hand, expression of the *Tert* gene in European hake (*Merluccius merluccius)* muscles increased with body length [39]. Although fish grow throughout their lives, there are cases of individuals whose growth rate definitely differs from the average in the population [50]. Growth disorder leading to dwarfism in fish is a condition that is observed in populations of fish living in the wild, as well as among farmed fish, especially from lines characterised by high inbreeding, e.g., androgenetic or gynogenetic fish [51, 52]. A rather rapid arrest of cell proliferation leading to the inhibition of somatic growth may result in a slower rate of telomeric sequence shortening in such fish. On the other hand, in such fish, the coexistence of malformations related to spinal deformities, which significantly hinder swimming, is often observed. The energy expenditure incurred by such fish is significantly higher than in the case of properly developing fish, as described in dwarf lake whitefish (*Coregonus clupeaformis*) [53]. Moreover, lake whitefish with dwarfism were characterised by a greater share of skeletal muscles and a relatively larger liver. [54]. A higher metabolic rate may have its consequences in the form of oxidative stress leading to faster shortening of telomeric DNA. Comparison of telomere length and telomerase activity in fish with dwarfism and their normally developing siblings is an intriguing idea that could bring new information about the role of telomerase in the somatic growth of fish and the consequences of this activity for the length of telomeric DNA.

Spontaneous and induced triploidisation of fish

In the case of a small number of fish species, triploid individuals have been observed to occur spontaneously in the wild. Lineages of gynogenetically reproducing fish that produce diploid gametes and lineages of fish that produce haploid gametes have been described in the pond loach (*Misgurnus anguillicaudatus*). Individuals from both lines can interbreed to produce triploid offspring [55–57]. Triploid females of this species from the clonal lineage lay haploid eggs, in which, after fertilisation by haploid sperm, diploid offspring developed [56]. In fish of the genus *Cobitis* also found in Poland, triploid hybrid individuals are observed

resulting from the crossing of fish belonging to the following species: Balkan loach (*C. elongatoides*), spined loach (*C. taenia*) and *C. tanaitica* [58]. Spontaneously appearing few-triploid individuals have been described in the case of populations in the wild and in breeding lineages of several species of salmonids, including rainbow trout [59–61]. Among more than 4,000 Atlantic salmon from 55 Norwegian farms, approximately 2% of the individuals turned out to be spontaneous triploids. The appearance of spontaneous triploids may be caused by the use for fertilisation of so-called overripe roe or roe that has been in the female body cavity for too long after ovulation. In such cases, after fertilisation, the second polar body is retained in the egg cell.

The studies described in this paper and concerning the dynamics of changes in telomeric DNA length and telomerase activity were carried out on diploid and triploid rainbow trout cells. Rainbow trout plays an important ecological role, it is one of the dominant species in the world's aquaculture, as well as a model organism in research on the formation of cancer, physiology, genetics and nutrition [62, 63]. Individuals with an additional set of chromosomes were obtained under controlled conditions by exposing fertilised eggs to an environmental shock, in this case a pressure shock, which by destabilising the action of the spindle apparatus microtubules prevents the second polar body from being ejected. As a consequence of this action, in the nucleus of the zygote there are three haploid sets of chromosomes; two sets of maternally inherited chromosomes from the female pronucleus (1n) and polar body (1n) and one paternally inherited set from the male pronucleus (1n) (Figure 2) [64]. The additional set of chromosomes in triploid fish causes serious disruption during gonad development and gamete production. In the case of salmonids, triploid females are functionally sterile, their ovaries are severely reduced, and the few oocytes they produce are aneuploid and unable to activate and develop normally. Triploid individuals do not mature and invest almost all energy from food in somatic development, thanks to which they are characterised by continuous growth, while in diploid fish during sexual maturation there is a significant reduction in the growth rate and even a decrease in body weight [64]. Triploid fish, because of their characteristic features such as larger cell size, increased heterozygosity (extra gene copies), sterility, continuous growth or greater susceptibility to external factors compared to diploid individuals, are a good model for the study of telomere length and telomerase activity. The extra set of chromosomes makes the regulation of gene expression in triploids an extremely interesting phenomenon, about which little is known, especially in the context of the *Tert* gene. Studies show that triploid individuals may be characterised by reduced, increased or similar expression of certain genes

[65–68]. The expression of the *Tert* gene is crucial for maintaining fish tissue homoeostasis, which is especially important for triploid individuals, which are more demanding in terms of appropriate environmental conditions compared to diploid fish.

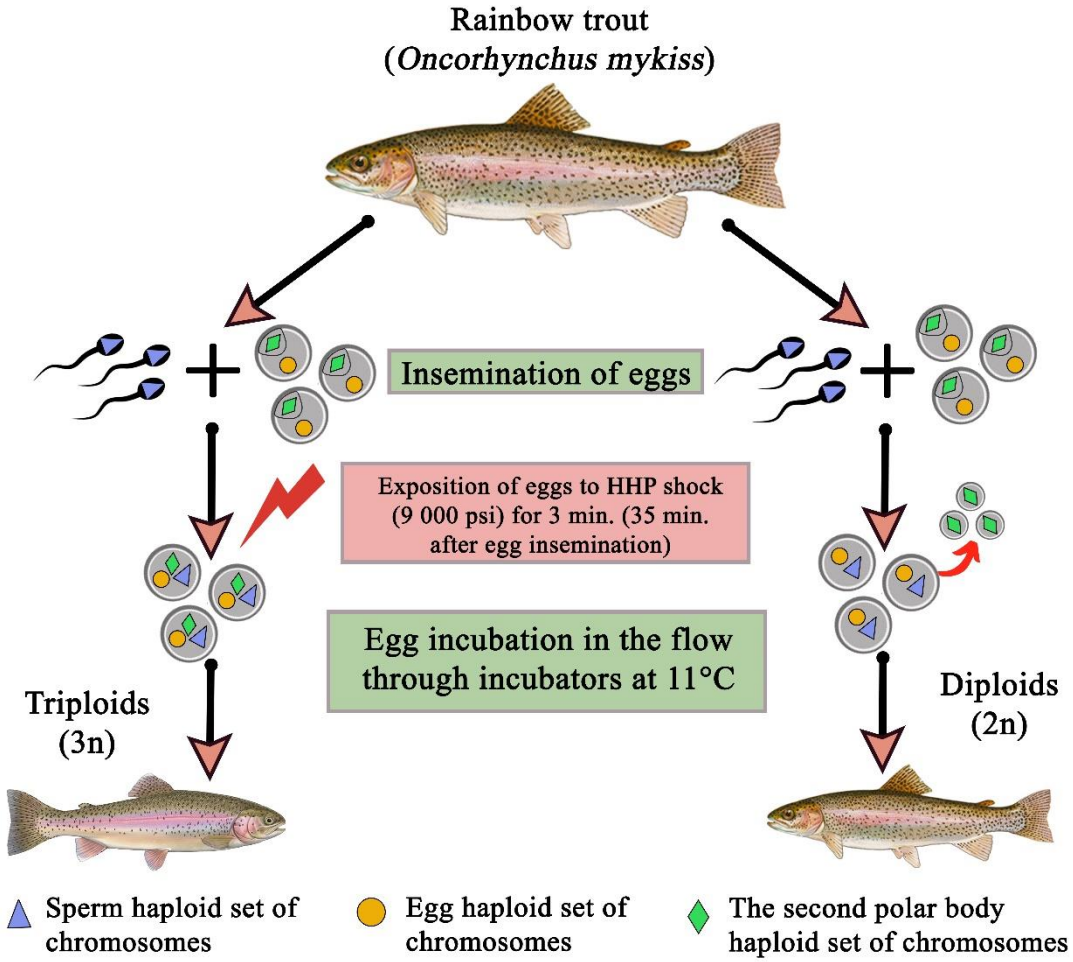


Figure 2 *The graphical summary of triploidisation induced in the rainbow trout*

Aim of the study

1. Description of telomeric DNA length changes during individual development in diploid and triploid rainbow trout cells.

2. Description of telomeric DNA length and telomerase activity in rainbow trout characterised by dwarfism.

3. Description of telomerase activity in somatic tissues and ovaries in diploid and triploid rainbow trout.

Research hypotheses

1. The length of telomeric DNA in diploid and triploid rainbow trout changes with age at different rates depending on ploidy.

2. Rainbow trout characterised by dwarfism have shorter telomeres and lower telomerase activity in cells than normally developed individuals.

3. Telomerase activity in the underdeveloped ovaries of triploid rainbow trouts is lower than in the ovaries of diploid individuals, while telomerase activity in the somatic organs of triploid rainbow trouts is higher than in diploid individuals.

Research tasks

To verify the hypotheses, the following research tasks were planned:

Task 1. Evaluation of telomeric DNA length changes in diploid and triploid rainbow trout cells of different ages.

Task 2. Analysis of telomeric DNA length and telomerase activity in dwarf and normally developing diploid rainbow trouts.

Task 3. Evaluation of the *Tert* gene expression activity in the liver, spleen, muscles, gills and ovaries of diploid and triploid rainbow trouts.

Verification of hypothesis 1.

The length of telomeric DNA in diploid and triploid rainbow trout changes with age at different rates depending on ploidy.

Telomeres in most mammals shorten with age, which is considered one of the mechanisms of replicative ageing [69]. Knowledge about the dynamics of changes in the length of telomeric DNA in fish concerns a very limited number of species, most of which studied in this respect are considered to be model species. The results obtained so far have allowed for the description of three variants of this phenomenon in fish: telomeric DNA shortens with age (1), the length of the $(TTAGGG)_n$ sequence is the same in fish of different ages (2) or it shortens and lengthens depending on the stage of individual development (3). An age-related decrease in telomere length has been observed in some strains of the Japanese rice fish and the turquoise killifish, but not in the case of *Menidia menidia* or the sea bass (*Dicentrarchus labrax*), in the cells of which telomeric DNA length is similar in juveniles and adults [37, 24, 70, 71]. In turn, in zebra fish, the telomeres first lengthen, reaching the maximum length observed in the cells of adult animals, and only then they gradually begin to shorten [28].

The aim of the study described in the first publication being part of the present doctoral thesis was to investigate how the length of telomeres changes in rainbow trout cells from diploid and triploid lineages at different stages of individual development. Triploid rainbow trout females have strongly reduced ovaries and do not invest energy in the gametogenesis process. For this reason, these fish do not mature sexually, and thus, unlike fertile diploid individuals, the growth rate of such fish does not decrease during the spawning season, which is commonly observed in the case of fertile females. Considering the physiological differences between trouts resulting from an additional haploid set of chromosomes, it was expected that the possible rate of shortening of telomeric DNA with age would also be different.

The length of $(TTAGGG)_n$ sequence in diploid and triploid rainbow trouts was analysed in cells of embryos, larvae, as well as one-year-old, two-year-old and three-year-old individuals. Interphase chromosomes were hybridised with a peptide nucleic acid probe labelled with fluorescein isothiocyanate (FITC) using the Telomere PNA FISH Kit/FITC (DAKO, Glostrup, Denmark). Then, using a camera (5 M CMOS), a microscopic image of interphase nuclei after hybridisation was taken and the intensity of fluorescent signals was

analysed using the Q-FISH (*Quantitative Fluorescence In Situ Hybridisation*) technique and HiFISH ASI software (Applied Spectral Imaging, Yokne'am Illit, Israel).

The results of the Q-FISH analysis showed that diploid and triploid fish are characterised by similar dynamics of telomeric DNA length changes during ontogenesis. The length of telomeres in the cells of embryos, larvae and one-year-old fish did not change significantly. Significantly shorter telomeres were found in two-year-old fish. Interestingly, the cells of three-year-old mice unexpectedly showed a significant increase in telomeric DNA length. In addition, it was observed that with the increase in weight and body length of triploid rainbow trouts, the length of telomeric DNA in their cells significantly decreases. Such a correlation has not been confirmed in diploid fish.

The decrease in telomere length observed in rainbow trout in the second year of life may be a result of the fish rapid growth from hatching to maturation. The results of studies conducted on numerous species of animals, including fish, confirm that the rate of telomere shortening is correlated with the period of rapid growth characteristic of the early stages of life of vertebrates [43]. The model of the dynamics of telomeric DNA changes in rainbow trout appears to be similar to that observed in the Japanese rice fish. In both species of fish, telomeres shorten and lengthen over the course of life depending on the rate of growth. Despite physiological and genetic differences, the rate of growth of diploid and triploid fish is similar to a certain point [65], only during the period of maturation and spawning, this rate in fertile individuals clearly decreases. For this reason, the dynamics of telomeric DNA length changes in fish from both groups is similar. During rapid growth accompanied by increased cell proliferation and thus increased free radical levels, telomerase was unable to compensate for the loss of telomeric DNA. The lengthening of telomeres in three-year-old fish may be related to the slowdown in growth rate during this period, which allowed for an increase in the length of telomeres in three-year old fish. The relationship between greater body weight/length and shorter telomeres observed in triploid fish cells confirms the results of studies showing a similar relationship in the American alligator [48]. The triploid fish studied in this case were significantly larger than the diploid fish, and perhaps this is the reason why only in the case of the former such a relationship appeared.

The obtained results allowed for a positive verification of the first part of the hypothesis, indicating that in diploid and triploid rainbow trout the length of telomeric DNA changes during ontogenesis, but ploidy did not affect the dynamics of these changes.

Verification of hypothesis 2

Rainbow trout characterised by dwarfism have shorter telomeres and lower telomerase activity in cells than normally developed individuals.

The rate of telomeric DNA shortening is greatest during the fastest growth characteristic of the early stages of vertebrate life [73–75]. Unlike warm-blooded organisms, ectothermic (cold-blooded) species are characterised by unrestricted growth, meaning they grow rapidly at a young age and continue to grow after reaching sexual maturity, but at a slower rate. In addition, the cells of somatic tissues of reptiles, amphibians and fish show a high level of telomerase expression, while in mammals and birds the activity of telomerase is clearly reduced in such cells [76, 77]. The correlation between body weight and telomerase activity in the liver, spleen and kidneys was confirmed by analysing several species of rodents. In species with an adult weight of less than one kilogram, telomerase activity was high in the studied tissues [78]. The level of telomerase activity in fish is characterised by high interspecies, intraspecies and individual variability [28, 36, 37, 79, 80]. The highest telomerase activity is usually found in cells of young and fast-growing individuals [39, 40]. Among the internal organs, high levels of telomerase have been described in the testes, ovaries and liver cells of zebra fish, turquoise killifish, southern platyfish (*Xiphophorus maculatus*) and rainbow trout [23, 24, 28, 36, 38, 80]. Moreover, in the case of turquoise killifish and European hake, male cells were characterised by higher telomerase activity [38, 39]. In the muscle tissue and skin of the European hake, a higher level of expression of the *Tert* gene was observed in individuals of greater weight. On the other hand, in adult rainbow trout individuals, the activity of telomerase in muscles decreased with increasing body weight. [36, 39]. Considering that in some species of fish such as European hake, rainbow trout and *Oryzias melastigma*, a relationship between somatic growth and telomere length or telomerase expression was observed [39, 40, 46], it seemed interesting to study telomerase activity and to determine telomere length in the cells of individuals with growth disorder (dwarfism) and those with a normal growth rate. Dwarfism is a condition that occurs quite often in the populations of numerous species of vertebrates, including fish. Growth disorder in dwarf individuals may be genetic or be a consequence of too low levels of growth hormone and malnutrition [81, 82]. Individuals characterised by reduced growth and body deformities have been described in populations of numerous wild fish species as well as those originating from farms (rainbow trout, Atlantic salmon, sea bass) [83]. In the case of rainbow trout, in lineages consisting of fully homozygous androgenetic fish, dwarf individuals are quite often observed,

and in their case this disorder is the result of the expression of recessive alleles [51, 52]. Comparison of telomerase activity and telomeric DNA length in the cells of individuals with growth disorder and fish with normal growth was the aim of the study in the next two articles included in the present doctoral thesis.

The length of telomeric DNA and telomerase activity were tested in one-year-old cells of normally developed androgenetic fish ($_{d}$DH), dwarf androgenotes ($_{d}$DH) and normally developed heterozygous fish from the Rutki lineage. Induced androgenesis is a procedure that allows for individuals inheriting only paternal chromosomes [84]. The process involves inactivating the roe by irradiating them with high doses of ionising or UV radiation, which destroy the nuclear DNA. Subsequently, the egg cells are inseminated, resulting in androgenetic haploid embryos. The next step is to subject the zygote to high hydrostatic pressure to stop the first division of the cell nucleus and duplicate the paternal genetic material. This results in so-called doubled haploids [84]. Some androgenotes are characterised by growth disorder or body deformities [51], which makes them good candidates for studies on the influence of growth disorders on telomeric DNA length and telomerase activity in fish. The length of telomeric DNA was tested in cells taken from the pronephros using the previously described Q-FISH method. In order to estimate the length of telomeric DNA, the fluorescence intensity of hybridisation signals in rainbow trout cells and mouse lymphoma cells from the *L5178Y-R* line of known telomere length (79,700 base pairs) was compared [85]. On the other hand, telomerase activity in liver, muscle and skin cells was tested using the ELISA TeloTAGGG Telomerase PCR ELISA Kit (Roche Diagnostics GmBH, Mannheim, Germany). It is a test designed for the highly sensitive detection of the activity of telomerase from biological samples.

A comparison of the length of the telomeric sequence in rainbow trout cells and L5178Y-R cells indicates that the average telomere length of the studied fish is approximately 20,000 base pairs, which is consistent with the observations of other scientists using the Southern Blot Hybridisation method to study the length of telomeric DNA [86]. The sex of the fish did not affect the length of the telomeric sequence. And most importantly, in the context of hypothesis verification, no statistically significant differences in telomeric DNA length were observed in rainbow trouts from the three study groups. The highest telomerase activity was observed in the liver cells of all tested fish. There were no statistically significant differences in telomerase activity in this organ in fish significantly different in length and weight. In muscles, telomerase activity was the lowest in heterozygous individuals. In

normally developed androgenetic individuals, compared to dwarf fish and heterozygous fish, an increased activity of telomerase in the skin was observed.

The similar length of telomeres in rainbow trouts with growth disorder and normally developed individuals suggests that the mechanisms associated with growth disorder do not affect the length of telomeric DNA. On the other hand, performed analyses demonstrated significant inter-individual variability in telomeric DNA length in rainbow trout of the same age. An equally large diversity of telomere lengths was observed in Japanese rice fish (from 6,000 base pairs to 12,000 base pairs) [38]. Studies involving mammals, but also various species of fish such as Japanese rice fish or turquoise killifish, show that females have longer telomeres than males [87, 88]. In turn, the results of the second publication demonstrated comparable length of telomeres in rainbow trout of both sexes, which is consistent with the results of the study of carp cells (*Cyprinus carpio*) [49]. Considering that the main task of telomerase is to limit excessive shortening of telomeres, comparable telomerase activity in the tissues of rainbow trouts with dwarfism and normally developed ones corresponds to the results described in the second publication, which showed no differences in telomere length between dwarf fish and the ones characterised by uninterrupted growth. The lack of significant differences in muscle telomerase activity in rainbow trouts with dwarfism and normal-sized individuals confirms that telomerase activity is not inhibited in dwarf individuals or that dwarfism is not a consequence of low telomerase activity. On the other hand, the reduced levels of telomerase observed in the skin of fish with growth disorder compared to normal androgenotes suggest that telomerase may be involved in growth-related processes in at least this tissue. Constant and high telomerase activity in fish tissues may be crucial in maintaining telomere homoeostasis during the regeneration process [23]. Studies conducted on zebra fish, Japanese rice fish and mummichog *(Fundulus heteroclitus)* have proven that the activity of telomerase in fish is related to their impressive ability to regenerate damaged tissues [79, 89–91]. Telomerase activity helps prevent telomere shortening during the rapid cell division that occurs during organ regeneration. In several species of fish, including rainbow trout, the liver is an organ with relatively high telomerase activity, which may be related to the ability to fully restore its function after damage [36, 38, 92].

To summarise, the telomeres of rainbow trouts with dwarfism were not shorter than those of normally developing fish, and telomerase activity in both groups of fish was similar, which allowed for the rejection of the hypothesis that rainbow trouts characterised by

dwarfism have shorter telomeres and lower telomerase activity in their cells than normally developed individuals.

Verification of hypothesis 3

Telomerase activity in the underdeveloped ovaries of triploid rainbow trouts is lower than in the ovaries of diploid individuals, while telomerase activity in the somatic organs of triploid rainbow trouts is significantly higher than in diploid individuals.

An additional set of chromosomes, and thus an increased number of alleles in triploid fish, makes such individuals more and more often the object of research in the field of regulation of gene expression [93, 94]. Because of their unique features such as: increased cell size and their lower number in the body, disturbed gonad development and gametogenesis, continuous growth, but also greater sensitivity to environmental conditions that deviate from the optimal ones compared to diploid individuals, triploid rainbow trouts are an interesting model in experiments on telomerase activity [64]. The additional set of chromosomes in triploid salmonids causes abnormal development of the gonads and interferes with oogenesis. As a result, female triploid rainbow trouts typically have underdeveloped ovaries with few oocytes, which prevents them from producing eggs and makes them sexually immature [95]. This functional sterility has its advantages, which are increasingly appreciated by the aquaculture sector; the growth rate of triploid females is not disturbed by processes related to reproduction, and the quality of muscle tissue does not decrease during the breeding season, which is often observed in diploid individuals during sexual maturation and spawning. The organs usually characterised by very high telomerase activity in fish are gonads [24, 28, 80]. Fish with the *Tert* gene turned off are characterised by premature infertility, atrophy of the gastrointestinal tract and loss of muscle mass (sarcopaenia) [41]. Considering that triploid salmonid females have an increased number of alleles, which may affect gene expression, and at the same time are sterile fish, the analysis of the *Tert* gene expression in the tissues of such fish seems to be extremely interesting from a scientific point of view. Therefore, the aim of the study presented in the fourth publication was to evaluate the expression of the *Tert* gene in somatic tissues and in the ovaries of diploid and triploid female rainbow trouts.

The *Tert* gene expression was tested in the liver, spleen, muscles, gills and ovaries of two- and three-year-old diploid and triploid female rainbow trouts. The RNA from these tissues was isolated using Bead-Beat Total RNA Mini kit (A&A Biotechnology, Gdańsk, Poland) and in further steps applied for cDNA synthesis with the use of the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, USA). Subsequently, a real-time PCR analysis was performed for the *Tert* gene and the *Actb* reference gene (β-actin). The ovaries of diploid and triploid females were examined macroscopically and then histology slides were

prepared, which were subsequently examined under a microscope. The results of Real-time PCR analysis showed that triploid individuals were characterised by significantly higher expression of the *Tert* gene in somatic tissues compared to diploid individuals. However, in the case of ovaries, a much higher level of expression of the *Tert* gene was observed in diploid fish. Expression of the *Tert* gene in muscles was higher in two-year-old fish only in the case of diploid individuals. In the gills, there were no significant differences in the activity of the *Tert* gene between the age groups in both diploid and triploid rainbow trouts. Three-year-old triploid fish were characterised by higher expression of *Tert* in the liver compared to two-year-old fish. Similar differences were not found in diploid rainbow trouts. In the spleen, a significant increase in the *Tert* gene activity occurred only in three-year-old triploid fish compared to diploids (regardless of age) and two-year-old triploid fish. The ovaries of triploid individuals were strongly reduced and composed mainly of connective tissue cells, most often fibrocytes, and contained few oocytes. The gonads of diploid fish were normally developed, and filled with oocytes at various stages of maturity.

The impact of triploidisation on gene expression has been studied, among others, in such species as Atlantic salmon, goldfish (*Carassius auratus*) or *Clarias macrocephalus* [96–98]. In the case of most of the genes analysed in these studies, the level of their expression in the tissues of diploid and triploid fish was similar. However, as a result of gene dosage compensation, some genes (e.g., genes regulating metabolic changes or stress response) may show a different level of expression in triploid individuals compared to diploids [68]. In the present study, triploid rainbow trouts, compared to diploids, were characterised by a higher level of the *Tert* expression in all investigated somatic tissues. This indicates that the *Tert* is subject to a gene dosage compensation mechanism associated with increased ploidy. Considering the differences between diploid and triploid rainbow trouts mentioned in the first paragraph, triploid individuals may require increased telomerase activity in order to maintain normal tissue homoeostasis. The liver, gills, spleen and muscles show significantly higher expression of telomerase in triploid rainbow trouts, which may be related to their physiology as well as contribute to their regenerative capacity and resistance to oxidative stress [64, 99]. The highest expression of the *Tert* gene observed in the liver may be related to its key role in metabolism, and thus greater exposure of the cells of this organ to reactive oxygen species and the impact of toxins [100]. Telomerase, apart from its basic function of maintaining the appropriate length of telomeres, also plays a role related to the elimination of free radicals [32]. Telomerase is also important in ovarian development and the production and maturation

of oocytes in fish. Zebra fish with the *Tert* gene turned off are characterised by gonadal atrophy, reduced egg production as well as premature infertility [41]. The presence of few oocytes, which are usually characterised by high telomerase activity, may be related to the observed reduction in *Tert* expression in the gonads of triploid rainbow trouts. In addition, telomerase is activated by oestrogens by stimulating the expression of the *Tert* gene, therefore the estradiol deficiency observed in the ovaries of triploid females may also be responsible for the reduced expression of the *Tert* [43].

The results of the study published in the fourth publication confirmed the third hypothesis, showing that the *Tert* expression in the underdeveloped ovaries of triploid rainbow trouts is lower than in the ovaries of diploid individuals while telomerase activity in the somatic organs of triploid rainbow trouts is higher than in diploid individuals.

Applied methods:

The research methodology included, among others:

- Carrying out the triploidisation process (Figure 2).
- Preparing interphase plates of cells from the pronephros of diploid, triploid and androgenetic rainbow trouts.
- Conducting Q-FISH analysis and microscopic analysis.
- Culturing the *L5178Y-R* cell line.
- Performing an ELISA test on selected tissues (liver, muscles, skin) from androgenetic and diploid rainbow trouts.
- Isolating total RNA from liver, spleen, muscles, gills and ovaries of diploid and triploid rainbow trouts of two and three years of age.
- Synthesising template DNA from RNA.
- Determining the expression level of the *Tert* gene using the Real-time PCR technique.

Summary

The average length of telomeric DNA in rainbow trout is approximately 20,000 base pairs, and females and males have similar telomere lengths. The length of the telomeric sequence in the cells of embryos, larvae and one-year-old fish did not change significantly. Significantly shorter telomeres were found in two-year-old rainbow trouts. In the cells of three-year-old animals, a significant increase in the length of telomeric DNA was found. Diploid and triploid rainbow trouts were characterised by similar dynamics of changes in telomeric DNA length, which suggests that differences at the molecular and physiological level resulting from an additional set of chromosomes do not significantly affect telomere length in this species.

The research results included in the second and third publications concerned the length of telomeres and telomerase activity in individuals with dwarfism. Dwarf rainbow trouts and normal-sized fish have comparable lengths of telomeric DNA and similar levels of telomerase activity in liver and muscles. In the case of the skin of dwarf fish, telomerase activity was lower compared to normally developed androgenetic individuals. The similar length of telomeres in rainbow trouts with growth disorder and normally developed individuals suggests that the mechanisms associated with growth disorder do not affect the length of telomeric DNA.

The aim of the research presented in the last publication was to determine the expression of the *Tert* gene in diploid and triploid rainbow trouts in different tissues. Triploid individuals were characterised by increased expression of the *Tert* in somatic tissues compared to diploid fish, which suggests that telomerase activity is crucial for maintaining tissue homoeostasis in fish with an additional set of chromosomes, which results, among others, in higher sensitivity to environmental conditions that deviate from the optimal ones. The situation is different in the ovaries; underdeveloped gonads of triploid females, built of connective tissue (mainly fibrocytes), which contained few oocytes, were characterised by a significantly reduced level of expression of the *Tert* gene. The gonads of diploid fish were normally developed, and filled with oocytes at various stages of maturity. The small number of reproductive cells, which are usually characterised by high telomerase activity, probably contributed to the low expression of the *Tert* gene observed in sterile ovaries.

Conclusions

1. The triploidisation process did not significantly affect the dynamics of telomeric DNA length changes during the individual development of rainbow trouts.
2. The rapid inhibition of somatic growth in rainbow trouts is not reflected in the length of telomeric DNA and telomerase activity.
3. Increased expression of the *Tert* gene in somatic cells in triploid rainbow trouts indicates an important function of telomerase in maintaining normal tissue function.
4. The reduced level of expression of the *Tert* gene in the underdeveloped ovaries of triploid females confirms the important role of telomerase in processes related to the development of gonads and fertility in fish.

Streszczenie

Telomery to znajdujące się na końcach chromosomów eukariotycznych konserwatywne regiony genomu składające się z krótkich i powtarzalnych sekwencji DNA (5'-TTAGGG-3') oraz białek (szelteryny). Telomery ulegają skracaniu podczas każdego podziału komórkowego, wykazując powiązanie z procesem starzenia się organizmu. Tempo skracania telomerowego DNA jest cechą osobniczą, na którą mają wpływ czynniki genetyczne oraz środowiskowe. Telomeraza jest rybonukleinoproteinowym enzymem, którego zadaniem jest synteza nici DNA w obszarze telomeru co spowalnia proces jego skracania się. Telomeraza składa się z dwóch kluczowych elementów: katalitycznej podjednostki *Tert* o charakterze odwrotnej transkryptazy (ang. *telomerase reverse transcript*ase), a także podjednostki TERC, która służy za wewnętrzną matrycę RNA. U większości organizmów stałocieplnych telomeraza nie wykazuje aktywności w komórkach somatycznych. Z kolei, u organizmów zmiennocieplnych, takich jak ryby aktywność telomerazy jest obserwowana w komórkach somatycznych przez cały okres trwania ich życia. Z tego względu telomerowy DNA u ryb nie zawsze skraca się wraz z wiekiem, a dynamika zmian długości telomerów może wyglądać inaczej u różnych gatunków. Ponadto, przypuszcza się, że stała ekspresja telomerazy u ryb jest związana między innymi z doskonałymi zdolnościami regeneracyjnymi tkanek oraz zwiększoną odpornością na choroby nowotworowe. Osobniki triploidalne ze względu na swoje unikalne cechy genetyczne i fizjologiczne: większe rozmiary komórek, wyższa heterozygotyczność, zahamowany rozwój gonad i ograniczona produkcja gamet, ciągły wzrost, a także obniżona odporność na niekorzystne warunki środowiskowe, są doskonałym modelem do badań dotyczących dynamiki zmian długości telomerowego DNA oraz aktywności telomerazy.

W niniejszej pracy zbadano zmiany długości telomerowego DNA, jak również ekspresję genu *Tert* oraz aktywność enzymu telomerazy w wybranych tkankach somatycznych i w gonadach u diploidalnych (2n) i triploidalnych (3n) pstrągów tęczowych (*Oncorhynchus mykiss*) w różnym wieku oraz u osobników charakteryzujących się odmiennym tempem wzrostu. Średnia długość sekwencji $(TTAGGG)_n$ jednorocznych pstrągów tęczowych wyniosła 20 000 par zasad. Różnice w długości telomerów u samic i samców były nieistotne. Badania wykazały, iż dynamika zmian długości telomerowego DNA u diploidalnych i triploidalnych ryb była podobna, co sugeruje, że dodatkowy zestaw chromosomów u ryb triploidalnych oraz wszelkie tego konsekwencje mają ograniczony

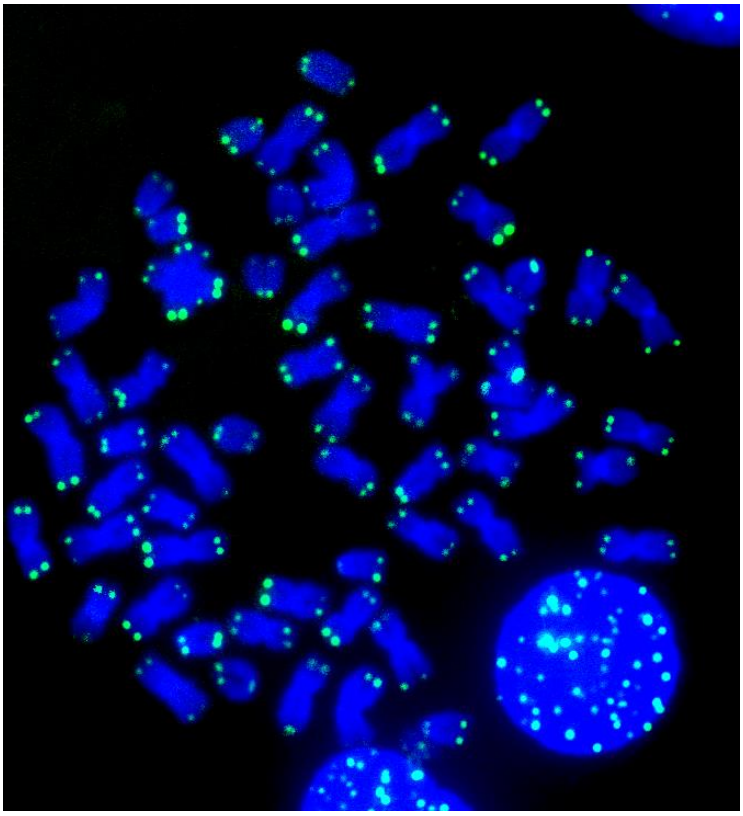
wpływ na długość telomerów u tego gatunku. W przypadku osobników z niedoborem wzrostu i prawidłowo rozwijających się nie wykazano istotnych różnic w długości telomerów. Aktywność telomerazy w wybranych tkankach osobników prawidłowo rozwijających się i karłowatych również nie różniła się zasadniczo, jedynie w skórze była niższa u ryb z niedoborem wzrostu. Podwyższony poziom ekspresji genu *Tert* stwierdzono w wątrobie, śledzionie, mięśniach oraz skrzelach osobników triploidalnych, co wydaje się mieć duże znaczenie w utrzymaniu homeostazy komórek u osobników, które w porównaniu do ryb diploidalnych są zdecydowanie bardziej wymagające środowiskowo. Natomiast w jajnikach ryb triploidalnych, ekspresja genu *Tert* była istotnie niższa w porównaniu do gonad diploidalnych samic. Jajniki triploidalnych pstrągów tęczowych były silnie zredukowane i zawierały nieliczne oocyty. Niewielka liczba komórek płciowych, które zazwyczaj charakteryzują się wysoką aktywnością telomerazy prawdopodobnie wpłynęła na obserwowaną w sterylnych jajnikach niską ekspresję genu *Tert*.

Słowa kluczowe: telomerowy DNA, telomeraza, pstrąg tęczowy, triploidyzacja, niedobór wzrostu

Wstęp

Telomery i telomeraza

Telomery to niekodujące regiony genomu składające się z tandemowo powtórzonych sekwencji TTAGGG, które wraz z kompleksem białek ochronnych (*ang.* shelterin - POT1, TPP1, TRF1, TRF2, RAP1, TIN2) są zlokalizowane na końcach chromosomów eukariotycznych (Rysunek 1) [1]. Telomery stabilizują strukturę chromosomów oraz zabezpieczają ich wewnętrzne regiony przed uszkodzeniem zawartej w nich informacji genetycznej podczas zachodzących podziałów komórkowych. Ponadto, telomery regulują ekspresję genów leżących w pobliżu regionu telomerowego, umożliwiają systemom naprawczym rozpoznanie zarówno prawidłowych, jak i uszkodzonych zakończeń chromosomów, zapobiegają mutacjom chromosomowym (translokacje, duplikacje, delecje) oraz zapewniają prawidłowy przebieg procesu rekombinacji i umożliwiają przestrzenną organizację jadra komórkowego [2-5].



Rysunek 1 *Chromosomy pstrąga tęczowego poddane hybrydyzacji z sondą telomerową PNA znakowaną izotiocyjanianiem fluoresceiny (FITC).*

U ludzi, jak i znacznej większości ssaków telomery skracają się wraz z wiekiem, co jest naturalną konsekwencją zachodzących podziałów komórkowych [6, 7]. Liniowe chromosomy eukariotyczne skracają się podczas replikacji, a zjawisko to nosi nazwę „problemu replikacji końca''. Ponieważ polimeraza DNA wymaga startera RNA do rozpoczęcia syntezy w kierunku 5' → 3', jedynie nić wiodąca DNA może być syntetyzowana do samego końca. W przypadku nici opóźnionej, której synteza odbywa się z wielu małych fragmentów DNA, nazywanych odcinkami Okazaki, nie może ona zostać w pełni zsyntetyzowana gdyż nie ma sposobu na syntezę ostatniego fragmentu Okazaki dlatego, że starter musiałby znajdować się poza końcem chromosomu. Przy tym przyłączony starter ostatniego odcinka Okazaki nie może zostać zastąpiony przez polimerazę DNA jak w przypadku pozostałych. W efekcie część sekwencji zlokalizowana na końcu replikowanego DNA pozostaje nieskopiowana, tworząc jednoniciową strukturę nazywaną z angielskiego *overhang* [8]. W wyniku tego procesu, z każdym cyklem komórkowym telomery ulegają stopniowemu skracaniu w tempie od 50 do 200 nukleotydów [9]. Trwa to, aż do momentu, gdy osiągną długość krytyczną, co jest komunikatem do zatrzymania podziałów komórkowych i rozpoczęcia procesu programowanej śmierci komórki (apoptozy). Skracanie się telomerowego DNA jest związane z procesem starzenia się, a także z rozwojem między innymi chorób nowotworowych, chorób układu krążenia, cukrzycy, chorób neurodegeneracyjnych (choroba Alzheimera) czy też chorób genetycznych (Zespół Wernera, Zespół Blooma) [10-12]. Postępująca utrata sekwencji telomerowej w komórkach somatycznych człowieka uważana jest za mechanizm supresji nowotworowej, natomiast niektóre komórki posiadają wadliwą odpowiedź na uszkodzenia DNA i kontynuują wzrost pomimo obecności dysfunkcyjnych telomerów [13]. Wyniki licznych badań wskazują, że stres oksydacyjny jest ważnym czynnikiem przyspieszającym tempo skracania się telomerów [14, 15]. Stres oksydacyjny definiuje się jako dysproporcję pomiędzy tempem wytwarzania reaktywnych form tlenu (RTF, ang. ROS – *reactive oxygen species*), a ich neutralizację przez systemy antyoksydacyjne [16]. Telomerowy DNA jest bogaty w nukleotydy guaninowe, które są szczególnie podatne na uszkodzenia powodowane oddziaływaniem reaktywnych form tlenu [14]. W przypadku zaburzenia homeostazy komórkowej, spowodowanej nadmierną produkcją RFT oraz upośledzeniem mechanizmów odpowiedzi na stres oksydacyjny, tempo skracania się telomerów może zostać zwiększone, co również przekłada się na tempo procesu starzenia się organizmu [15]. Reaktywne formy tlenu są produkowane podczas procesów metabolicznych, ale ich poziom może wzrosnąć w wyniku narażenia na czynniki takie jak: wysoko przetworzona dieta uboga w antyoksydanty, stres, promieniowanie UV czy

zanieczyszczenie środowiska [15, 17, 18]. Wszystkie z wymienionych czynników mają negatywny wpływ na utrzymanie odpowiedniej długości telomerów. Średnia długość sekwencji telomerowej jest cechą charakterystyczną dla gatunku, jednak różnice w długości telomerów i tempie ich skracania się zaobserwowano także między osobnikami tego samego gatunku. Dodatkowo, długość telomerowego DNA może być różna w komórkach poszczególnych tkanek/narządów tego samego organizmu. Na przykład u ludzi długość sekwencji $(TTAGGG)_n$ mieści się w zakresie od około 5 000 to 15 000 nukleotydów [19]. Zauważono, że długość telomerowego DNA jest inna w zależności od płci, przy czym kobiety mają zazwyczaj dłuższe telomery niż mężczyźni [20, 21]. Być może jest to spowodowane występowaniem u kobiet wyższych poziomów estrogenów, które wykazują działania przeciwzapalne i antyoksydacyjne.

Badania dotyczące chromosomowej lokalizacji sekwencji telomerowej wykonano u około 80 gatunków ryb. Długość telomerowego DNA u ryb mieści się w zakresie od około 3 000 par zasad (drętwa pawik (*Torpedo ocellata*)) do nawet 25 000 par zasad (danio pręgowany (*Danio rerio*)) [22, 23]. Telomery u ryb podobnie jak u ludzi mogą skracać się wraz z wiekiem, co zaobserwowano u zagrzebki (*Nothobranchius furzeri*), jesiotra syberyjskiego (*Acipenser baeri*) lub gambuzji (*Gambusia affinis*) [24-26]. Z drugiej strony, długość telomerów buffalo czarnego (*Ictiobus cyprinellus*) nie zmienia się w ciągu życia [27]. Jeszcze inaczej wygląda to u danio pręgowanego, w komórkach którego telomerowy DNA wydłuża się i skraca w zależności od etapu rozwoju na jakim w danym momencie znajduje się ryba [28]. Badania przeprowadzone na cierniczku (*Pungitius pungitius*) wykazały, że u samic tego gatunku skracanie się telomerów postępuje wraz z osiągnięciem dojrzałości płciowej, co sugeruje, że procesy dojrzewania płciowego wymagające większych nakładów energetycznych u samic mogą prowadzić do wystąpienia stresu oksydacyjnego, a w konsekwencji przyspieszenia skracania się telomerowego DNA. Takich zmian nie zaobserwowano u samców tego gatunku [29].

Telomeraza jest rybonukleinoproteinowym enzymem, który odgrywa kluczową rolę w utrzymaniu długości i integralności telomerów. Enzym ten jest odpowiedzialny za syntezę sekwencji telomerowego DNA, spowalniając lub zapobiegając skracaniu się telomerów. Telomeraza składa się z podjednostki katalitycznej *Tert* o charakterze odwrotnej transkryptazy (ang. *telomerase reverse transcript*ase), podjednostki TERC złożonej z cząsteczki RNA będącej matrycą podczas syntezy telomerowego DNA oraz niezbędnego do

zachowania przestrzennej struktury telomerów białka dyskeryny [1, 30]. Oprócz utrzymania odpowiedniej długości telomerów, telomeraza odgrywa znaczącą rolę między innymi podczas regeneracji uszkodzonych tkanek, w procesie nowotworzenia, ochronie antyoksydacyjnej czy też mechanizmie starzenia się organizmu [31, 32]. U większości organizmów stałocieplnych aktywność telomerazy została potwierdzona jedynie w komórkach linii płciowej, komórkach macierzystych oraz komórkach nowotworowych [31]. Badania pokazują jednak, że ekspresja telomerazy może zostać indukowana podczas regeneracji tkanek lub gojenia ran [33]. Co ciekawe, u myszy z wyłączonym genem *Tert* dochodzi do utraty zdolności regeneracji tkanek, w tym skóry, co objawia się wypadaniem i siwieniem włosów oraz zmniejszoną zdolnością gojenia się ran [34, 35]. W przeciwieństwie do ssaków, u których aktywność telomerazy jest w znacznej mierze ograniczona, telomeraza u ryb jest aktywna w komórkach wszystkich tkanek bez względu na wiek badanych osobników, co opisano u kilkunastu gatunków [23, 24, 28, 36-40]. Badania na modelowych gatunkach ryb pokazują wysoką korelację pomiędzy ekspresją genu *Tert*, a aktywnością telomerazy, co sugeruje, że regulacja transkrypcji tego genu jest jednym z podstawowych mechanizmów regulujących aktywność enzymu [40]. Pierwszym gatunkiem ryb, u którego potwierdzono tak powszechną aktywność telomerazy, także u starszych osobników był pstrąg tęczowy [36]. U pozbawionych aktywności telomerazy danio pręgowanych *(Tert*$^{-/-}$*)* zaobserwowano przedwczesną bezpłodność, atrofię tkanek, utratę masy ciała oraz nasilenie stanów zapalnych [41]. Zagrzebki z wyłączonym przy pomocy techniki CRISPR/Cas9 genem *Tert* charakteryzowały się obniżoną płodnością oraz występowaniem atroficznych jąder i jajników [42]. Wydaje się zatem, że wysoka aktywność telomerazy u ryb oprócz oczywistych funkcji związanych z kontrolą długości telomerów, zapewnia także rybom utrzymanie homeostazy organów i tkanek.

Zależność między długością telomerów oraz aktywnością telomerazy, a masą ciała ryb jest złożoną kwestią. Długość telomerowego DNA jest cechą genetycznie determinowaną, ale dynamika zmian długości sekwencji $(TTAGGG)_n$ jest wypadkową tempa wzrostu, liczby podziałów komórkowych, narażenia na stres oksydacyjny i aktywności telomerazy, która w przypadku organizmów zmiennocieplnych jest obecna w komórkach somatycznych [43]. Podstawową funkcją telomerazy jest dobudowywanie nukleotydów do sekwencji telomerowej w każdym cyklu podziału komórkowego, co może wyjaśniać brak korelacji między długością telomerów, a wiekiem u ogrodowca zwyczajnego (*Thamnophis sirtalis*), żółwia skórzastego (*Dermochelys coriacea*) [44, 45] czy kilku gatunków ryb, gdzie długość telomerów u dorosłych osobników jest porównywalna do tych zaobserwowanych u młodych ryb [27].

Ale nie jest to regułą. W przypadku ryb, gwałtowne tempo wzrostu od wyklucia do osiągnięcia dojrzałości płciowej może prowadzić do skracania się telomerów, co obserwuje się w komórkach kilku badanych pod tym kątem gatunków [37, 46]. Szybko rosnące transgeniczne łososie pacyficzne (*Oncorhynchus kisutch*) z dodatkowymi kopiami genu hormonu wzrostu cechują się krótszymi telomerami w porównaniu do nietransgenicznych ryb z grupy kontrolnej. Co więcej, w okresie gwałtownego wzrostu, tempo skracania się telomerowego DNA u transgenicznych osobników było zdecydowanie szybsze [47]. Wzmożona produkcja wolnych rodników towarzysząca intensywnemu wzrostowi ryb może powodować wystąpienie stresu oksydacyjnego, który z kolej przyczynia się do zwiększenia tempa „erozji" telomerów. Wydaje się zatem, że telomeraza nie zawsze jest w stanie zrekompensować stratę telomerowego DNA będącą konsekwencją intensywnej proliferacji komórek podczas szybkiego wzrostu somatycznego. Trudno też szukać jednego modelu zależności między długością telomerów, a wielkością czy masą ciała u zwierząt zmiennocieplnych. U aligatora amerykańskiego (*Alligator mississippiensis*) osobniki o większej długości ciała posiadały krótsze telomery [48]. Z kolei u karpia, długość telomerowego DNA zwiększała się wraz z długością ciała ryb [49]. Aktywność telomerazy w komórkach mięśniowych może być relatywnie niska u dorosłych ryb, które siłą rzeczy mają większą masę ciała niż młode osobniki, co obserwuje się u pstrąga tęczowego i dorsza [36, 39]. Z drugiej strony, ekspresja genu *Tert* w mięśniach morszczuka (*Merluccius merluccius*) rosła wraz z długością ciała osobników [39]. Mimo, że ryby rosną przez całe swoje życie, to zdarzają się przypadki osobników, których tempo wzrostu zdecydowanie odbiega od średniej w populacji [50]. Niedobór wzrostu prowadzący do karłowatości u ryb jest stanem, który obserwuje się w populacjach dziko-żyjących osobników, a także wśród ryb hodowlanych, szczególnie z linii charakteryzujących się dużą wsobnością, np. ryby androgenetyczne czy gynogenetyczne [51, 52]. Dosyć gwałtowne zatrzymanie proliferacji komórek prowadzące do zahamowania wzrostu somatycznego może powodować, że tempo skracanie się sekwencji telomerowej u takich ryb będzie wolniejsze. Z drugiej strony, u tego rodzaju ryb obserwuje się często współistnienie wad rozwojowych związanych z deformacjami kręgosłupa utrudniającymi w znacznym stopniu pływanie. Wydatek energetyczny jaki ponoszą takie ryby jest znacząco wyższy niż w przypadku ryb prawidłowo rozwijających się, co opisano u karłowatych siei (*Coregonus clupeaformis*) [53]. Co więcej, sieje z niedoborem wzrostu charakteryzowały się większym udziałem mięśni szkieletowych oraz stosunkowo większą wątrobą [54]. Wyższe tempo przemiany materii może mieć swoje konsekwencje w postaci wystąpienia stresu oksydacyjnego prowadzącego do szybszego skracania się telomerowego DNA.

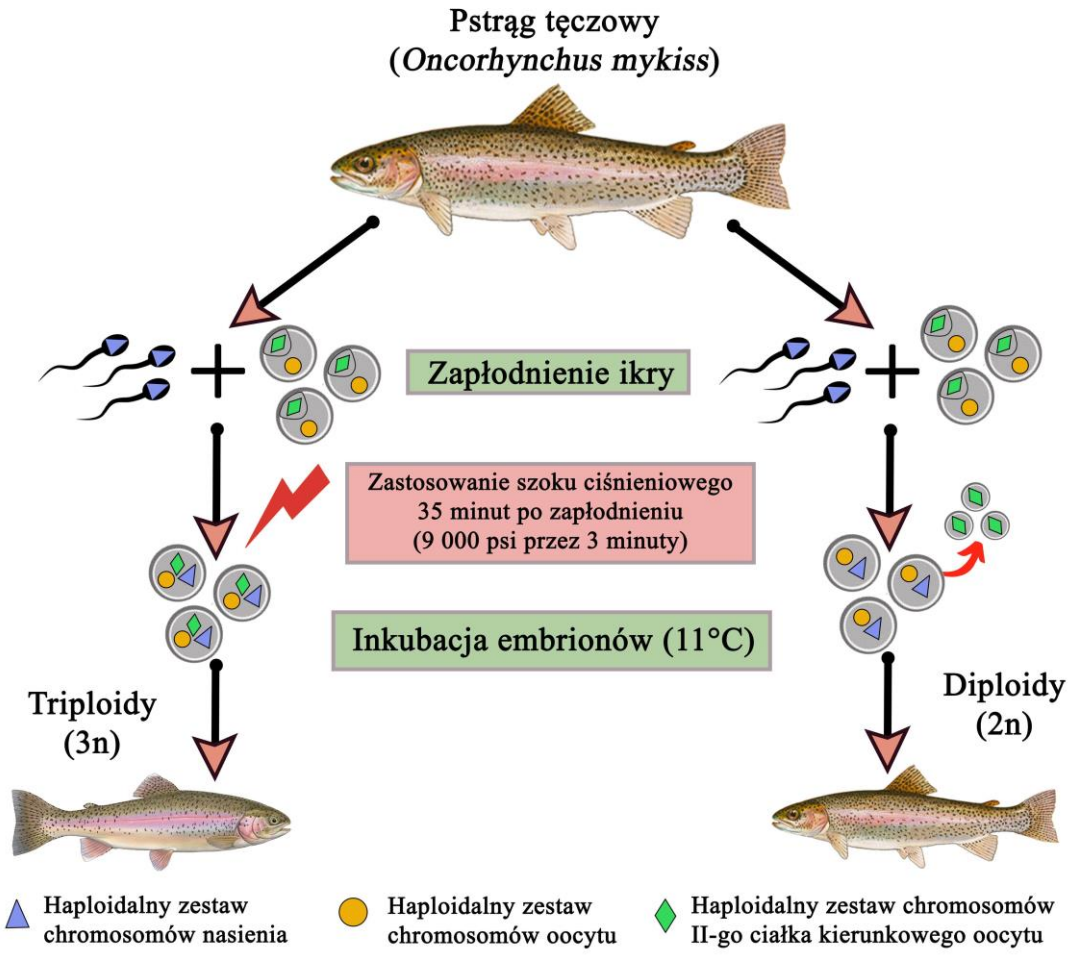
Porównanie długości telomerów i aktywności telomerazy u ryb z niedoborem wzrostu oraz ich prawidłowo rozwijającego się rodzeństwa wydaje się intrygującym pomysłem mogącym przynieść nowe informacje dotyczące roli telomerazy podczas wzrostu somatycznego ryb oraz konsekwencji tejże aktywności dla długość telomerowego DNA.

Spontaniczna i indukowana triploidyzacja ryb

W przypadku niewielkiej liczby gatunków ryb zaobserwowano spontaniczne występowanie w środowisku naturalnym osobników triploidalnych. U piskorza amurskiego (*Misgurnus anguillicaudatus*) opisano linie ryb rozmnażających się gynogenetycznie, które produkują diploidalne gamety oraz linie ryb, które produkują gamety haploidalne. Osobniki z obu linii mogą się ze sobą krzyżować dając triploidalne potomstwo [55-57]. Triploidalne samice tego gatunku z linii klonalnej składają haploidalną ikrę, w której po zapłodnieniu przez haploidalne plemniki rozwinęło się diploidalne potomstwo [56]. U ryb z rodzaju *Cobitis* występujących również w Polsce obserwuje się triploidalne osobniki hybrydowe powstałe w wyniku krzyżowania się ryb należących do gatunków: koza dunajska *(C. elongatoides)*, koza *(C. taenia)* oraz *C. tanaitica* [58]. Spontanicznie pojawiające się i nieliczne triploidalne osobniki opisano w przypadku dziko-żyjących populacji i linii hodowlanych kilku gatunków ryb łososiowatych w tym także w przypadku pstrąga tęczowego [59-61]. Wśród ponad 4 000 łososi atlantyckich pochodzących z 55 norweskich farm około 2% osobników okazało się być spontanicznymi triploidami. Pojawienie się spontanicznych triploidów może być spowodowane wykorzystaniem do zapłodnienia tak zwanej przejrzałej ikry, czyli ikry która zbyt długo po owulacji przebywała w jamie ciała samicy. W takich przypadkach, po zapłodnieniu dochodzi do zatrzymaniem drugiego ciałka kierunkowego w komórce jajowej.

Badania opisane w niniejszej pracy i dotyczące dynamiki zmian długości telomerowego DNA oraz aktywności telomerazy przeprowadzono na komórkach diploidalnych i triploidalnych pstrągów tęczowych. Pstrąg tęczowy pełni ważną rolę ekologiczną, jest jednym z dominujących gatunków w światowej akwakulturze, jak również jest organizmem modelowym w badaniach dotyczących powstawania nowotworów, fizjologii, genetyki czy też żywienia [62, 63]. Osobniki z dodatkowym zestawem chromosomów uzyskano w warunkach kontrolowanych eksponując zapłodnione ziarna ikry na działanie udaru środowiskowego, w tym przypadku szoku ciśnieniowego, który destabilizując działanie mikrotubul wrzeciona kariokinetycznego zapobiega wyrzuceniu drugiego ciałka kierunkowego. W konsekwencji takiego działania, w jądrze zygoty znajdują się trzy haploidalne zestawy chromosomów; dwa komplety chromosomów dziedziczonych po

matce pochodzące z przedjądrza żeńskiego (1n) i ciałka kierunkowego (1n) oraz jeden komplet dziedziczony po ojcu pochodzący z przedjądrza męskiego (1n) (Rysunek 2) [64]. Dodatkowy zestaw chromosomów u triploidalnych ryb powoduje poważne zakłócenia podczas rozwoju gonad i produkcji gamet. W przypadku ryb łososiowatych, triploidalne samice są funkcjonalnie sterylne, ich jajniki są silnie zredukowane, a produkowane przez nie nieliczne oocyty są aneuploidalne i niezdolne do aktywacji i prawidłowego rozwoju. Triploidalne osobniki nie dojrzewają i inwestują niemal całą energię z pokarmu w rozwój somatyczny, dzięki czemu charakteryzują się ciągłym wzrostem, podczas gdy u ryb diploidalnych w okresie dojrzewania płciowego dochodzi do znaczącego ograniczenia tempa wzrostu, a nawet zmniejszenia masy ciała [64]. Ryby triploidalne ze względu na swoje charakterystyczne cechy takie jak większy rozmiar komórek, zwiększona heterozygotyczność (dodatkowe kopie genów), sterylność, ciągły wzrost czy też większa podatność na czynniki zewnętrzne w porównaniu do diploidalnych osobników, są dobrym modelem do badań długości telomerów oraz aktywności telomerazy. Dodatkowy zestaw chromosomów powoduje, że regulacja ekspresji genów u triploidów jest niezwykle ciekawym zjawiskiem, o którym wciąż niewiele wiemy zwłaszcza w kontekście genu *Tert*. Badania pokazują, iż osobniki triploidalne mogą charakteryzować się zmniejszoną, zwiększoną lub podobną ekspresją niektórych genów [65-68]. Ekspresja genu *Tert* jest kluczowa dla utrzymania homeostazy tkanek ryb, co jest szczególnie istotne dla osobników triploidalnych, które są bardziej wymagające w kontekście odpowiednich warunków środowiskowych w porównaniu do diploidalnych ryb.



Rysunek 2 *Graficzne przedstawienie procesu triploidyzacji pstrąga tęczowego.*

Cele badań

1. Charakterystyka zmian długości telomerowego DNA w trakcie rozwoju osobniczego w komórkach diploidalnych i triploidalnych pstrągów tęczowych.

2. Określenie długości telomerowego DNA i aktywności telomerazy u pstrągów tęczowych charakteryzujących się niedoborem wzrostu.

3. Określenie aktywności telomerazy w tkankach somatycznych i w jajnikach u diploidalnych i triploidalnych pstrągów tęczowych.

Hipotezy badawcze

1. Długość telomerowego DNA u diploidalnych i triploidalnych pstrągów tęczowych zmienia się wraz z wiekiem w różnym tempie, zależnie od ploidalności.

2. Pstrągi tęczowe charakteryzujące się zaburzeniem wzrostu posiadają krótsze telomery oraz niższą aktywność telomerazy w swoich komórkach niż prawidłowo rozwinięte osobniki.

3. Aktywność telomerazy w niedorozwiniętych jajnikach triploidalnych pstrągów tęczowych jest niższa, niż w jajnikach osobników diploidalnych, natomiast aktywność telomerazy w organach somatycznych triploidalnych pstrągów tęczowych jest wyższa niż u diploidalnych osobników.

Zadania badawcze

Weryfikacji hipotez podporządkowano następujące zadania badawcze:

Zadanie 1. Oszacowanie zmian długości telomerowego DNA w komórkach diploidalnych i triploidalnych pstrągów tęczowych w różnym wieku.

Zadanie 2. Analiza długości telomerowego DNA oraz aktywności telomerazy u karłowatych i prawidłowo rozwijających się diploidalnych pstrągów tęczowych.

Zadanie 3. Ocena aktywności ekspresji genu *Tert* w wątrobie, śledzionie, mięśniach, skrzelach i jajnikach diploidalnych i triploidalnych pstrągów tęczowych.

Weryfikacja hipotezy 1.

Długość telomerowego DNA u diploidalnych i triploidalnych pstrągów tęczowych zmienia się wraz z wiekiem w różnym tempie, zależnie od ploidalności.

Telomery u większości ssaków skracają się wraz z wiekiem, co jest uważane za jeden z mechanizmów starzenia replikacyjnego [69]. Wiedza dotycząca dynamiki zmian długości telomerowego DNA u ryb odnosi się do bardzo ograniczonej liczby gatunków, z których większość przebadanych pod tym kątem to gatunki uznane za modelowe. Dotychczas uzyskane wyniki pozwoliły opisać trzy warianty tego fenomenu u ryb: telomerowy DNA skraca się wraz z wiekiem (1), długość sekwencji $(TTAGGG)_n$ jest taka sama u ryb w różnym wieku (2) lub skraca się i wydłuża w zależności od etapu rozwoju osobniczego (3). Związany z wiekiem spadek długości telomerów został zaobserwowany w przypadku niektórych szczepów ryżanki japońskiej oraz zagrzebki, natomiast nie wykazano takiej zależności w przypadku *Menidia menidia* czy okonia morskiego (*Dicentrarchus labrax*), w komórkach których długość telomerowego DNA u osobników młodocianych i dorosłych jest podobna [37, 24, 70, 71]. Z kolei u danio pręgowanego telomery najpierw wydłużają się osiągając maksymalną długość obserwowaną w komórkach dorosłych osobników i dopiero potem stopniowo zaczynają się skracać [28].

Celem badań opisanych w pierwszej publikacji składającej się na niniejszą pracę doktorską było zbadanie jak zmienia się długość telomerów w komórkach pstrągów tęczowych z diploidalnych i triploidalnych linii na różnych etapach rozwoju osobniczego. Triploidalne samice pstrąga tęczowego posiadają silnie zredukowane jajniki i nie inwestują energii w proces gametogenezy. Z tego też powodu ryby te nie dojrzewają płciowo, a co za tym idzie w przeciwieństwie do płodnych diploidalnych osobników tempo wzrostu takich ryb nie spada w sezonie tarłowym co jest powszechnie obserwowane w przypadku płodnych samic. Biorąc pod uwagę fizjologiczne różnice między pstrągami tęczowymi wynikające z posiadania dodatkowego haploidalnego zestawu chromosomów oczekiwano, że ewentualne tempo skracania się telomerowego DNA wraz z wiekiem także będzie różne.

Długość sekwencji $(TTAGGG)_n$ u diploidalnych i triploidalnych pstrągów tęczowych analizowano w komórkach zarodków, larw, osobników jednorocznych, dwuletnich oraz trzyletnich. Chromosomy interfazowe poddano hybrydyzacji z sondą telomerową PNA (ang. *peptide nucleic acid probe*) znakowaną izotiocyjanianiem fluoresceiny (FITC) używając zestawu Telomere PNA FISH Kit/FITC (DAKO, Glostrup, Dania). Następnie z

wykorzystaniem kamery (5 M CMOS) pobrano obraz mikroskopowy jąder interfazowych po hybrydyzacji i przeanalizowano intensywność sygnałów fluorescencyjnych wykorzystując technikę Q-FISH (ang. *Quantitative Fluorescence In Situ Hybridization*) i program HiFISH ASI (Applied Spectral Imaging, Yokne'am Illit, Israel).

Wyniki analizy Q-FISH wykazały, że ryby diploidalne i triploidalne charakteryzują się podobną dynamiką zmian długości telomerowego DNA w trakcie ontogenezy. Długość telomerów w komórkach zarodków, larw i jednorocznych ryb nie zmieniała się istotnie. Znacząco krótszymi telomerami charakteryzowały się dwuletnie ryby. Co ciekawe, niespodziewanie w komórkach trzyletnich osobników stwierdzono znaczny wzrost długości telomerowego DNA. Ponadto zaobserwowano, że wraz ze wzrostem masy oraz długości ciała triploidalnych pstrągów tęczowych istotnie spada długość telomerowego DNA w ich komórkach. Takiej korelacji nie potwierdzono u ryb diploidalnych.

Spadek długości telomerów obserwowany u pstrągów tęczowych w drugim roku życia może być wynikiem szybkiego wzrostu występującego u ryb od wyklucia do etapu dojrzewania. Wyniki badań przeprowadzonych na wielu gatunkach zwierząt, w tym także na rybach, potwierdzają, że tempo skracania się telomerów jest skorelowane z okresem szybkiego wzrostu charakterystycznego dla początkowych faz życia kręgowców [43]. Model dynamiki zmian telomerowego DNA u pstrąga tęczowego wydaje się być podobny do tego zaobserwowanego u ryżanki japońskiej. U obu gatunków ryb telomery skracają się i wydłużają w trakcie życia w zależności od tempa wzrostu. Pomimo różnic fizjologicznych i genetycznych tempo wzrostu ryb diploidalnych i triploidalnych do pewnego momentu jest podobne [65], dopiero w okresie dojrzewania i samego tarła to tempo u płodnych osobników wyraźnie spada. Z tego też powodu dynamika zmian długości telomerowego DNA u ryb z obu grup jest podobna. Podczas gwałtownego wzrostu, któremu towarzyszy wzmożona proliferacja komórek, a tym samym zwiększa się poziom wolnych rodników, telomeraza nie była w stanie zrekompensować ubytków telomerowego DNA. Wydłużenie telomerów u ryb trzyletnich może być związane ze spowolnieniem tempa wzrostu w tym okresie, co pozwoliło telomerazie na zwiększenie długości telomerów u trzylatków. Obserwowana w komórkach triploidalnych ryb zależność większa masa/długość ciała – krótsze telomery potwierdza wyniki badań pokazujących podobne powiązanie u aligatora amerykańskiego [48]. Osobniki triploidalne, które badano w tym przypadku były istotnie większe od ryb diploidalnych i być może stąd jedynie w przypadku tych pierwszych taka zależność się pojawiła.

Uzyskane wyniki pozwoliły na pozytywną weryfikacje pierwszej części hipotezy, wskazując, że u diploidalnych oraz triploidalnych pstrągów tęczowych długość telomerowego DNA zmienia się w trakcie trwania ontogenezy, jednak ploidalność nie miała wpływu na dynamikę tych zmian.

Weryfikacja hipotezy 2

Pstrągi tęczowe charakteryzujące się zaburzeniem wzrostu posiadają krótsze telomery oraz niższą aktywność telomerazy w swoich komórkach niż prawidłowo rozwinięte osobniki.

Tempo skracania telomerowego DNA jest największe w trakcie najszybszego wzrostu charakterystycznego dla wczesnych etapów życia kręgowców [73-75]. W przeciwieństwie do organizmów stałocieplnych, gatunki ektotermiczne (zmiennocieplne) charakteryzują się nieograniczonym wzrostem co oznacza, że rosną szybko w młodym wieku i kontynuują wzrost po osiągnięciu dojrzałości płciowej, lecz w wolniejszym tempie. Ponadto w komórkach tkanek somatycznych gadów, płazów oraz ryb wykazano wysoki poziom ekspresji telomerazy, podczas gdy u ssaków i ptaków aktywność telomerazy jest wyraźnie ograniczona w takich komórkach [76, 77]. Korelację między masą ciała i aktywnością telomerazy w wątrobie, śledzionie oraz w nerkach potwierdzono analizując kilkanaście gatunków gryzoni. U gatunków, których masa dorosłego osobnika jest niższa niż jeden kilogram, aktywność telomeraza była wysoka w badanych tkankach [78]. Poziom aktywności telomerazy u ryb cechuje duża międzygatunkowa, wewnątrzgatunkowa, a także osobnicza zmienność [28, 36, 37, 79, 80]. Najwyższą aktywnością telomerazy zazwyczaj charakteryzują się komórki młodych i szybko rosnących osobników [39, 40]. Wśród organów wewnętrznych wysoki poziom telomerazy opisano w komórkach jąder, jajników i wątroby u danio, ryżanki japońskiej, zagrzebki, zmienniaków plamistych (*Xiphophorus maculatus*) oraz u pstrąga tęczowego [23, 24, 28, 36, 38, 80]. Co więcej, w przypadku ryżanki japońskiej i morszczuka, wyższą aktywnością telomerazy charakteryzowały się komórki samców [38, 39]. W tkance mięśniowej i w skórze morszczuka wyższy poziom ekspresji genu *Tert* zaobserwowano u osobników o większej masie. Z kolei u dorosłych osobników pstrąga tęczowego wraz ze wzrostem masy ciała spadała aktywności telomerazy w mięśniach. [36, 39]. Biorąc pod uwagę, że u niektórych gatunków ryb takich jak morszczuk, pstrąg tęczowy oraz *Oryzias melastigma* zaobserwowano związek między wzrostem somatycznym i długością telomerów lub ekspresją telomerazy [39, 40, 46] interesującym wydawało się zbadanie aktywności telomerazy oraz określenie długości telomerów w komórkach osobników z niedoborem wzrostu (karłowatość) i tych charakteryzujących się prawidłowym tempem wzrostu. Karłowatość to przypadłość, która występuje dosyć często w populacjach wielu gatunków zwierząt kręgowych włączając w to ryby. Niedobór wzrostu u osobników karłowatych może mieć podłoże genetyczne lub być konsekwencją zbyt niskiego poziomu hormonu wzrostu oraz niedożywienia [81, 82]. Osobniki charakteryzujące się zredukowanym wzrostem i

deformacjami ciała opisano w populacjach wielu gatunków ryb dziko żyjących jak również tych pochodzących z hodowli (pstrąg tęczowy, łosoś atlantycki, okoń morski) [83]. W przypadku pstrąga tęczowego, w liniach składających się z w pełni homozygotycznych ryb androgenetycznych dosyć często obserwuje się osobniki karłowate, w przypadku których to zaburzenie jest efektem ekspresji recesywnych alleli [51, 52]. Porównanie aktywności telomerazy oraz długości telomerowego DNA w komórkach osobników z niedoborem wzrostu i ryb charakteryzujących się prawidłowym wzrostem było celem badań, w kolejnych dwóch artykułach wchodzących w skład niniejszej pracy doktorskiej.

Długość telomerowego DNA i aktywność telomerazy zbadano w komórkach jednorocznych prawidłowo rozwiniętych ryb androgenetycznych ($_{n}$DH), karłowatych androgenotów ($_{d}$DH) oraz prawidłowo rozwiniętych heterozygotycznych ryb z linii Rutki. Indukowana androgeneza jest zabiegiem pozwalającym otrzymać osobniki dziedziczące jedynie ojcowskie chromosomy [84]. Proces ten polega na inaktywacji ikry poprzez naświetlenie wysokimi dawkami promieniowania jonizującego lub UV, które niszczą jądrowy DNA. Następnie komórki jajowe poddane są inseminacji w wyniku czego uzyskuje się androgenetyczne haploidalne zarodki. Kolejnym etapem jest poddanie zygoty na działanie wysokiego ciśnienia hydrostatycznego w celu zatrzymania pierwszego podziału jądra komórkowego i zduplikowania ojcowskiego materiału genetycznego. W rezultacie otrzymuje się tak zwane podwojone haploidy (ang. *Doubled Haploids*) [84]. Niektóre androgenoty charakteryzują się niedoborem wzrostu, czy też deformacjami ciała [51], co czyni je dobrymi kandydatami do badań nad wpływem zaburzeń wzrostu na długość telomerowego DNA oraz aktywności telomerazy u ryb. Długość sekwencji telomerowej badano w komórkach pobranych z nerki głowowej stosując wcześniej opisaną metodę Q-FISH. W celu oszacowania długości telomerowego DNA porównano intensywność fluorescencji sygnałów hybrydyzacji w komórkach pstrąga tęczowego i w mysich komórkach chłoniaka z linii *L5178Y-R* o znanej długości telomerów (79 700 par zasad) [85]. Natomiast aktywność telomerazy w komórkach wątroby, w mięśniach i skórze zbadano posługując się testem ELISA TeloTAGGG Telomerase PCR ELISA Kit (Roche Diagnostics GmBH, Mannheim, Niemcy). Jest to test przeznaczony do wysoce czułego wykrywania aktywności telomerazy z próbek biologicznych.

Porównanie długości sekwencji telomerowej w komórkach pstrągów tęczowych i komórkach L5178Y-R wskazuje, że średnia długość telomerów badanych ryb wynosi około 20 000 par zasad, co jest zgodne z obserwacjami innych naukowców wykorzystujących do

badań długości telomerowego DNA metodę hybrydyzacji typu Southern blot (ang. *Southern blot hybridisation*) [86]. Na długość sekwencji telomerowej nie miała wpływu płeć ryb. I co najistotniejsze w kontekście weryfikacji hipotezy, nie zaobserwowano statystycznie istotnych różnic w długości telomerowego DNA u pstrągów tęczowych z trzech badanych grup. Najwyższą aktywność telomerazy zaobserwowano w komórkach wątroby u wszystkich badanych ryb. Nie stwierdzono statystycznie istotnych różnic w aktywności telomerazy w tym organie u ryb znacząco różniących się długością i masą. W mięśniach aktywność telomerazy była najniższa u osobników heterozygotycznych. U prawidłowo rozwiniętych osobników androgenetycznych w porównaniu do ryb karłowatych i ryb heterozygotycznych zaobserwowano podwyższoną aktywność telomerazy w skórze.

Podobna długość telomerów u pstrągów tęczowych z zaburzeniem wzrostu jak i normalnie rozwiniętych osobników sugeruje, że mechanizmy związane z niedoborem wzrostu nie wpływają na długość telomerowego DNA. Z drugiej strony, przeprowadzone analizy pokazały znaczącą międzyosobniczą zmienność długości telomerowego DNA u pstrągów tęczowych będących w tym samym wieku. Równie dużą różnorodność długości telomerów zaobserwowano w komórkach ryżanki japońskiej (od 6000 par zasad do 12000 par zasad) [38]. Badania z udziałem ssaków, ale też różnych gatunków ryb takich jak ryżanka japońska lub zagrzebka pokazują że samice posiadają dłuższe telomery niż samce [87, 88]. Podczas gdy wyniki drugiej publikacji wskazały porównywalną długości telomerów u pstrągów tęczowych obu płci, co jest z kolei zgodne z wynikami badań komórek karpia (*Cyprinus carpio*) [49]. Biorąc pod uwagę, że głównym zadaniem telomerazy jest ograniczenie nadmiernego skracania się telomerów, porównywalna aktywność telomerazy w tkankach pstrągów tęczowych o ograniczonym wzroście i normalnie rozwiniętych osobników koresponduje z wynikami opisanymi w drugiej publikacji, które pokazały brak różnic w długości telomerów między rybami karłowatymi, a tymi o niezakłóconym wzroście. Brak istotnych różnic w aktywności telomerazy w mięśniach u pstrągów tęczowych z deficytem wzrostu i osobników o prawidłowej budowie potwierdza, że aktywności telomerazy nie jest hamowana u osobników karłowatych lub, że karłowatość nie jest konsekwencją niskiej aktywności telomerazy. Z drugiej strony, obniżony poziom telomerazy obserwowany w skórze ryb z niedoborem wzrostu w porównaniu do normalnych androgenotów sugeruje, że telomeraza może być zaangażowana w procesy związane ze wzrostem przynajmniej w tej tkance. Stała i wysoka aktywność telomerazy w tkankach ryb może być kluczowa w utrzymaniu homeostazy telomerów podczas procesu regeneracji [23]. Badania

przeprowadzone na danio pręgowanym, ryżance japońskiej oraz przydence żebrowatej (*Fundulus heteroclitus*) udowodniły, że aktywność telomerazy u ryb jest powiązana z ich imponującymi możliwościami regeneracyjnymi uszkodzonych tkanek [79, 89-91]. Aktywność telomerazy pomaga zapobiegać skracaniu telomerów podczas szybkiego podziału komórek, które zachodzą w trakcie regeneracji narządów. U kilku gatunków ryb w tym także u pstrąga tęczowego, wątroba jest organem o stosunkowo wysokiej aktywności telomerazy, co może być powiązane ze zdolnościami do pełnego przywrócenia funkcji po jej uszkodzeniu [36, 38, 92].

Reasumując, telomery pstrągów tęczowych z zaburzeniami wzrostu nie były krótsze od telomerów ryb prawidłowo rozwijających się, a aktywność telomerazy w obu grupach ryb była podobna co pozwoliło odrzucić hipotezę mówiącą o tym, że pstrągi tęczowe charakteryzujące się zaburzeniem wzrostu, posiadają krótsze telomery oraz niższą aktywność telomerazy w swoich komórkach niż prawidłowo rozwinięte osobniki.

Weryfikacja hipotezy 3

Aktywność telomerazy w niedorozwiniętych jajnikach triploidalnych pstrągów tęczowych jest niższa, niż w jajnikach osobników diploidalnych, natomiast aktywność telomerazy w organach somatycznych triploidalnych pstrągów tęczowych jest znacząco wyższa niż u diploidalnych osobników.

Dodatkowy zestaw chromosomów, a co za tym idzie zwiększona liczba alleli u triploidalnych ryb powoduje, że takie osobniki coraz częściej są obiektem badań w zakresie regulacji ekspresji genów [93, 94]. Ze względu na swoje unikalne cechy takie jak: zwiększona wielkość komórek i ich mniejsza liczba w organizmie, zakłócony proces rozwoju gonad i gametogenezy, ciągły wzrost, ale także większa wrażliwość na odbiegające od optymalnych warunki środowiskowe w porównaniu do osobników diploidalnych, triploidalne pstrągi tęczowe są ciekawym modelem w doświadczeniach dotyczących aktywności telomerazy [64]. Dodatkowy zestaw chromosomów u triploidalnych ryb łososiowatych powoduje nieprawidłowy rozwój gonad oraz zakłóca proces oogenezy. W rezultacie samice triploidalnych pstrągów tęczowych zazwyczaj mają niedorozwinięte jajniki z niewielką liczbą oocytów, co uniemożliwia im produkcję jaj i powoduje, że ryby te nie dojrzewają płciowo [95]. Ta funkcjonalna sterylność ma swoje zalety doceniane coraz częściej przez sektor akwakultury; tempo wzrostu triploidalnych samic nie jest zakłócone przez procesy związane z rozrodczością, a jakość tkanki mięśniowej nie spada w sezonie rozrodczym, co często obserwuje się u osobników diploidalnych w trakcie dojrzewania płciowego i tarła. Organami charakteryzującymi się zazwyczaj bardzo wysoką aktywnością telomerazy u ryb są gonady [24, 28, 80]. Ryby z wyłączonym genem *Tert* cechują się przedwczesną bezpłodnością, atrofią przewodu pokarmowego, a także utratą masy mięśniowej (sarkopenia) [41]. Biorąc pod uwagę, że triploidalne samice ryb łososiowatych posiadają zwiększoną liczbę alleli, co może mieć wpływ na ekspresję genów, a przy tym są rybami sterylnymi, analiza ekspresji genu *Tert* w tkankach takich ryb wydaje się z naukowego punktu widzenia niezwykle ciekawa. W związku z tym, celem badań przedstawionych w czwartej publikacji było oszacowanie ekspresji genu *Tert* w tkankach somatycznych oraz w jajnikach diploidalnych i triploidalnych samic pstrąga tęczowego.

Ekspresja genu *Tert* została zbadana w wątrobie, śledzionie, mięśniach, skrzelach oraz jajnikach dwuletnich i trzyletnich diploidalnych oraz triploidalnych samic pstrąga tęczowego. RNA z wymienionych tkanek zostało wyizolowane przy użyciu zestawu Bead-Beat Total RNA Mini kit (A&A Biotechnology, Gdańsk, Polska) i w dalszych etapach wykorzystane do

syntezy cDNA stosując RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, USA). Następnie przeprowadzono analizę real-time PCR dla genu *Tert* oraz genu referencyjnego *Actb (*β-aktyna). Jajniki diploidalnych i triploidalnych samic badano makroskopowo oraz sporządzono z nich preparaty histologicznie, które następnie badano pod mikroskopem. Wyniki analizy real-time PCR wykazały, że osobniki triploidalne charakteryzowały się znacząco wyższą ekspresją genu *Tert* w tkankach somatycznych w porównaniu do osobników diploidalnych. Natomiast w przypadku jajników, zdecydowanie wyższy poziom ekspresji genu *Tert* zaobserwowano u ryb diploidalnych. Ekspresja genu *Tert* w mięśniach była większa u ryb dwuletnich tylko w przypadku diploidalnych osobników. W skrzelach nie odnotowano istotnych różnic w aktywności genu *Tert* między grupami wiekowymi zarówno u diploidalnych jak i u triploidalnych pstrągów tęczowych. Trzyletnie ryby triploidalne charakteryzowały się wyższą ekspresją *Tert* w wątrobie w porównaniu do ryb dwuletnich. Podobnych różnic nie wykazano u pstrągów tęczowych diploidalnych. W śledzionie istotny wzrost aktywności genu *Tert* wystąpił tylko u trzyletnich osobników triploidalnych w porównaniu do diploidów (bez względu na wiek) oraz dwuletnich ryb triploidalnych. Jajniki osobników triploidalnych były silnie zredukowane oraz zbudowane głównie z komórek tkanki łącznej, najczęściej fibrocytów i zawierały nieliczne oocyty. Gonady ryb diploidalnych były prawidłowo rozwinięte, wypełnione oocytami na różnym stopniu dojrzałości.

Wpływ triploidyzacji na ekspresję genów był badany między innymi u takich gatunków jak łosoś atlantycki, karaś chiński (*Carassius auratus*) czy *Clarias macrocephalus* [96-98]. W przypadku większości badanych w tych pracach genów poziom ich ekspresji w tkankach ryb diploidalnych i triploidalnych był zbliżony. Natomiast w wyniku kompensacji dawki genów (ang. *dosage compensation*) niektóre geny (na przykład geny regulujące przemiany metaboliczne lub odpowiedź na stres) mogą wykazywać inny poziom ekspresji u osobników triploidalnych w porównaniu do diploidów [68]. W niniejszej pracy triploidalne pstrągi tęczowe w porównaniu do diploidów charakteryzowały się wyższym poziomem ekspresji genu *Tert* we wszystkich badanych tkankach somatycznych. Wskazuje to, że *Tert* podlega mechanizmowi kompensacji dawki genów związanemu z podwyższoną ploidalnością. Biorąc pod uwagę wspomniane w pierwszym akapicie różnice między diploidalnymi, a triploidalnymi pstrągami tęczowymi, osobniki triploidalne mogą wymagać zwiększonej aktywności telomerazy w celu utrzymania właściwej homeostazy tkanek. Wątroba, skrzela, śledziona i mięśnie wykazują istotnie wyższą ekspresję telomerazy u

triploidalnych pstrągów tęczowych, co może być związane z ich fizjologią, jak również przyczyniać się do ich zdolności regeneracyjnych oraz odporności na stres oksydacyjny [64, 99]. Najwyższa ekspresja genu *Tert* obserwowana w wątrobie może mieć związek z jej kluczową rolą w metabolizmie, a co za tym idzie większym narażeniem komórek tego organu na reaktywne formy tlenu i działanie toksyn [100]. Telomeraza poza swoją podstawową funkcją jaką jest utrzymanie odpowiedniej długości telomerów, pełni też rolę związaną z eliminacją wolnych rodników [32]. Telomeraza ma również duże znaczenie w rozwoju jajników i produkcji oraz dojrzewaniu oocytów u ryb. Danio pręgowane z wyłączonym genem *Tert* charakteryzują się atrofią gonad, zmniejszoną produkcją jaj, a także przedwczesną niepłodnością [41]. Obecność niewielu oocytów, które zazwyczaj charakteryzują się wysoką aktywnością telomerazy, może mieć związek z obserwowanym zmniejszeniem ekspresji *Tert* w gonadach triploidalnych pstrągów tęczowych. Poza tym, telomeraza jest aktywowana przez estrogeny poprzez stymulację ekspresji genu *Tert*, dlatego też niedobór estradiolu obserwowany w jajnikach triploidalnych samic może również być odpowiedzialny za obniżoną ekspresję *Tert* [43].

Wyniki badań zamieszczone w czwartej publikacji potwierdziły hipotezę trzecią, wykazując że w niedorozwiniętych jajnikach triploidalnych pstrągów tęczowych ekspresja genu *Tert* jest niższa niż w jajnikach osobników diploidalnych, natomiast aktywność telomerazy w organach somatycznych triploidalnych pstrągów tęczowych jest wyższa niż u diploidalnych osobników.

Stosowane metody:

Metodologia badawcza obejmowała między innymi:

- Przeprowadzenie procesu triploidyzacji (Rysunek 2).
- Wykonanie preparatów płytek interfazowych komórek pochodzących z nerki głowowej diploidalnych, triploidalnych oraz androgenetycznych pstrągów tęczowych.
- Przeprowadzenie analizy Q-FISH oraz analizy mikroskopowej.
- Hodowla linii komórkowej *L5178Y-R.*
- Wykonanie testu ELISA na wybranych tkankach (wątroba, mięśnie, skóra) pochodzących od androgenetycznych i diploidalnych pstrągów tęczowych.
- Izolacja całkowitego RNA z wątroby, śledziony mięśni, skrzeli oraz jajników diploidalnych oraz triploidalnych pstrągów tęczowych w wieku dwóch i trzech lat.
- Synteza matrycowego DNA z RNA.
- Określenie poziomu ekspresji genu *Tert* przy zastosowaniu techniki real-time PCR.

Podsumowanie

Średnia długość telomerowego DNA u pstrąga tęczowego wynosi około 20 000 par zasad, a samice oraz samce cechowały się zbliżoną długością telomerów. Długość sekwencji telomerowej w komórkach zarodków, larw i jednorocznych ryb nie zmieniała się istotnie. Znacząco krótszymi telomerami charakteryzowały się dwuletnie pstrągi tęczowe. W komórkach trzyletnich osobników stwierdzono znaczny wzrost długości telomerowego DNA. Diploidalne i triploidalne pstrągi tęczowe charakteryzowały się podobną dynamiką zmian długości telomerowego DNA, co sugeruje, że różnice na poziomie molekularnym i fizjologicznym będące konsekwencją dodatkowego zestawu chromosomów nie wpływają znacząco na długość telomerów u tego gatunku.

Wyniki badań zamieszczone w drugiej i trzeciej publikacji dotyczyły długości telomerów oraz aktywności telomerazy u osobników z niedoborem wzrostu. Karłowate pstrągi tęczowe i ryby z prawidłowym wzrostem posiadają porównywalną długość telomerowego DNA oraz podobny poziom aktywności telomerazy w wątrobie i mięśniach. W przypadku skóry ryb karłowatych aktywność telomerazy była mniejsza w porównaniu z normalnie rozwiniętymi osobnikami androgenetycznymi. Zbliżona długość telomerów u pstrągów tęczowych z zaburzeniem wzrostu jak i prawidłowo rozwiniętych osobników sugeruje, że mechanizmy związane z niedoborem wzrostu nie wpływają na długość telomerowego DNA.

Celem badań przedstawionych w ostatniej publikacji było określenie ekspresji genu *Tert* u diploidalnych oraz triploidalnych pstrągów tęczowych w różnych tkankach. Osobniki triploidalne charakteryzowały się podwyższoną ekspresją *Tert* w tkankach somatycznych w porównaniu do ryb diploidalnych, co sugeruje, że aktywność telomerazy jest kluczowa dla utrzymania homeostazy tkanek u ryb posiadających dodatkowy zestaw chromosomów czego konsekwencją jest między innymi wyższa wrażliwość na odbiegające od optymalnych warunki środowiskowe. Inaczej sytuacja przedstawia się w jajnikach; niedorozwinięte gonady triploidalnych samic zbudowane z tkanki łącznej (głównie fibrocytów), które zawierały nieliczne oocyty charakteryzowały się zdecydowanie obniżonym poziomem ekspresji genu *Tert*. Gonady ryb diploidalnych były prawidłowo rozwinięte, wypełnione oocytami na różnym stopniu dojrzałości. Niewielka liczba komórek płciowych, które zazwyczaj charakteryzują się wysoką aktywnością telomerazy prawdopodobnie wpłynęła na obserwowaną w sterylnych jajnikach niską ekspresję genu *Tert*.

Wnioski

1. Proces triploidyzacji nie wpłynął znacząco na dynamikę zmian długości telomerowego DNA w trakcie rozwoju osobniczego pstrągów tęczowych.
2. Gwałtowane zahamowanie wzrostu somatycznego u pstrągów tęczowych nie ma swojego odzwierciedlenia w długości telomerowego DNA i aktywności telomerazy.
3. Podwyższona ekspresja genu *Tert* w komórkach somatycznych u triploidalnych pstrągów tęczowych wskazuje na ważną funkcję telomerazy w utrzymaniu prawidłowego funkcjonowania tkanek.
4. Obniżony poziom ekspresji genu *Tert* w niedorozwiniętych jajnikach triploidalnych samic potwierdza istotną rolę telomerazy w procesach związanych z rozwojem gonad i płodnością u ryb.

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Telomere Dynamics in the Diploid and Triploid Rainbow Trout (*Oncorhynchus mykiss*) Assessed by Q-FISH Analysis

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Abstract: Changes of telomere length with age were assessed in diploid and triploid rainbow trout (*Oncorhynchus mykiss*) females in the cross-sectional study using Q-FISH technique. Triploid trout as sterile do not invest an energy in gametogenesis and continue to grow, whereas fertile diploid individuals suffer from declines in growth and survival during sexual maturation. However, triploid and diploid specimens exhibited similar patterns of telomere dynamics. Telomere length in the embryos, larvae and one-year-old juveniles did not change significantly. In the second year after hatching, subadults exhibited substantially shortened telomeres, while significant increase of the telomere length was reported in the three-year-old adults. On the other hand, correlation between telomere length and body size was observed in the triploid, but not in the diploid rainbow trout. Telomere shortening observed in two-year-old subadults may have been associated with the premature period of the fast growth in rainbow trout. Similar pattern of the telomere dynamics reported in the fertile diploids and sterile triploids indicated processes related to reproduction did not affect telomere dynamics in this species. Unexpected increase of the telomere length reported during the third year of life confirmed that in rainbow trout telomeric DNA shortens and lengthens, depending on the developmental stage.

Keywords: aging; growth; telomere attrition

1. Introduction

Telomeres are nucleoprotein complexes at the ends of eukaryotic chromosomes. In all vertebrates studied to date, the DNA component of the telomeres contains tandemly repeated G-rich hexanucleotide sequence $(TTAGGG/CCCTAA)_n$ [1]. Telomeres protect chromosomes from end-to-end fusions and degradation, guarantee their complete replication and allow DNA repair machinery to distinguish natural chromosomal ends from the ends that appear in the course of breakage events [2,3]. As the DNA polymerase is not able to replicate ends of linear chromosomes ("end replication problem") telomeric DNA shorten with every cell division [4]. Moreover, attrition of telomeres is accelerated by the oxidative stress associated with increased production of reactive oxygen species (ROS) [5]. ROS are generated during aerobic metabolism and ATP production in mitochondria. Moreover, ROS are formed in the course of exposure to the UV light, ionizing radiation and xenobiotics [6]. Rich in guanine telomeric DNA is particularly susceptible to damage from ROS and many experiments confirmed that oxidative stress increases incidences of the DNA strand breaks leading to loss of distal telomere fragments [7]. The process of telomere loss may be compensated mainly by the telomerase, an enzyme

whose catalytic protein subunit (TERT, telomerase reverse transcriptase) adds telomeric DNA repeats to the end of telomeres using as a template an integral RNA component (TR, telomerase RNA). In contrast to humans, where telomerase expression in the adults is restricted to germ line cells, stem cells and tumors, expression of telomerase in various ectotherm species has been detected in many tissues irrespective of age [8–10].

Consistent decline of the telomere length with age has usually been observed in the endotherms [11]. In ectotherms results concerning relationship between age and telomere length are not that equivocal [12]. Age-related telomere shortening has been confirmed in alligators (*Alligator mississippiensis*) [13] and garter snakes (*Thamnophis elegans*) [14], but not in the leatherback turtle (*Dermochelys coriacea*) [15]. In fish, age-dependent decline of telomeres has been reported in some strains of Japanese medaka (*Oryzias latipes*) [16] and killifish (*Nothobranchius furzeri*) [17], but no gradual attrition of telomeric DNA with age was evidenced in the European sea bass (*Dicentrarchus labrax*) [18] or Atlantic silverside (*Menidia menidia*) [19]. In turn, in zebrafish (*Danio rerio*), the telomere length increases from larvae to the adult stage and shorten significantly in the aged individuals [20].

Somatic growth and reproduction are energetically costly and generate large amount of ROS and both processes have been found to affect dynamics of telomere attrition [21]. Taking into account that energetic investments in the growth and reproduction vary between males and females, intersexual differences in the telomere length should not be surprising. Indeed, in many species studied under this regard, females live longer than males and also have longer telomeres [22]. Recent experiments show that due to the estrogens, mitochondria from females produces less reactive oxygen species than males [23]. Moreover, in the egg producing animals, vitellogenin that is an egg yolk precursor has also been found to act as an antioxidant [24].

Although, processes related to reproduction are likely to matter in the context of telomere length, little attention has been devoted to analyzing telomere dynamics in the triploid sterile fish. The additional set of chromosomes causes cytogenetic incompatibility, which makes most of the triploids unable to reproduce. Triploid fish do not invest an energy in gametogenesis and continue to grow whereas normal diploid specimens suffer from declines in growth and survival during sexual maturation [25,26]. Spontaneous occurrence of triploids was confirmed in several fish species [27–29]. Moreover, methods of artificial triploidization has been elaborated for many commercially important aquaculture fishes [30]. Currently, triploids are used in the production of rainbow trout (*Oncorhynchus mykiss* Walbaum, 1792)—one of the most important salmonid fish species in world aquaculture [25]. Although, rainbow trout was the first fish with confirmed multitissue activity of telomerase irrespective of the individual age [8], to date, no information concerning alterations of the telomere length with age has been provided in this species. Therefore, the main goal of the present research was to examine changes of the telomere length in fertile diploid and sterile triploid rainbow trout sampled at different developmental stages from embryogenesis to adulthood. To assess the dynamics of telomeres, sensitive and specific quantitative fluorescence in situ hybridization (Q-FISH) method has been utilized [31,32]. This technique includes the application of fluorescein-conjugated peptide nucleic acid (PNA) telomere probe that stains telomeres proportionally to their length and the software that enables capturing and quantification of the fluorescent signals. Q-FISH has been successfully applied to interphase nuclei to quantify change in the telomere length with age in several species including fish [20].

2. Materials and Method

This study was carried out in strict accordance with the recommendations in the Polish ACT of 21 January 2005 of Animal Experiments (Dz. U. of. 2005 No 33, item 289). The protocol was approved by the Local Ethical Committee for the Experiments on Animals in Bydgoszcz.

2.1. Fish Origin

Embryos at 16 day post-fertilization (dpf), larvae (28 dpf), juvenile (one-year-old), subadult (two-year-old) and adult (three-year-old) females from the diploid and triploid stocks of rainbow trout from Rutki strain, reared in the Department of Salmonid Research (DSR), Inland Fisheries Institute in Olsztyn (IFI), Rutki, Poland were sampled for examination of the telomere length dynamics. No fish older than three years were available. Triploid stocks from the Department of Salmonid Research were produced using standard procedure including application of high hydrostatic pressure (HHP) shock (9000 psi/3 min.) to the fertilized eggs 35 min. after insemination (Dobosz, personal communication).

2.2. Egg Incubation and Fish Rearing

Diploid and triploid rainbow trout were reared separately, but under the same husbandry and environmental conditions. The hatchery and the outdoor grow-out facilities were supplied with water from Radunia River that average temperature was 11 °C (range: winter 0–4 °C, spring 4–16 °C, summer 15–22 °C, autumn 5–14 °C). Eggs were incubated in the conventional flow through incubators. After hatching, larvae at the swim-up stage were transferred to the plastic tanks (1 m^3) where they were reared during the first year of life. Within their second and third year of life, diploid and triploid fish were kept outdoor in the rectangular (10 m^3) and rotational concrete ponds (56 m^3), respectively. Fish were fed daily and feeding rates were adjusted to the growth and daily temperatures. When water temperature was between 18 and 20 °C feeding was reduced to half. Feeding was fully stopped when water temperature was higher than 20 °C. During the entire experiment, fish were held under natural light conditions.

2.3. Preparation of Interphase Spreads

In the present research, eight embryos, eight larvae and five juveniles, six subadults and five adults randomly chosen from each, diploid and triploid stock were examined. The sample size was determined to meet requirements for the statistical analysis, based on the previous studies in fish [20]. All sampled trout individuals were sacrificed by an overdose of propiscin (*Etomidatum*, IFI (Inland Fisheries Institute, Olsztyn, Poland), weighed and their length measured. In the diploid and triploid juveniles, subadults and adults, development of reproductive organs and presence of oocytes were assessed macroscopically after fish dissection.

Suspensions of cells from embryos and larvae were prepared with the modified method described by Polonis et al. [33]. Briefly, full larvae and embryos that had been gently removed from the eggs were incubated in 0.075-M KCl solution for 30 min at room temperature and then transferred to the tubes with 50 µL of fixative (methanol: acetic acid, 3:1), which was changed twice. The tissue was left in the fixative for 20 min. Heads of the fixed embryos and larvae were cut off and macerated with scissors and dissociated by pipetting in 60 µL of methanol–acetic acid (2:1) to obtain homogeneous cell suspensions. Single drops of cell suspension from each individual were dropped on the microscope slides and left to dry at room temperature for 24 h.

The somatic cells of juveniles, subadults and adults were prepared from the head kidney tissue. Head kidney is a primary fish lymphoid and endocrine organ usually used for preparation of metaphase and interphase spreads for the cytogenetic analysis including fluorescence in situ hybridization (FISH) with PNA (peptide nucleic acid) telomere probe [34,35]. Portions of the head kidney were placed in tubes with 5 mL of KCl (0.075 M), homogenized and left for 40 min at room temperature. Next, 10 drops of freshly prepared ice cold fixative (methanol: acetic acid, 3:1) were added. After 2 min, the tubes were filled with the fixative up to 10 mL and centrifuged at 160× *g* for 10 min. Then, the supernatant was tossed out and fresh fixative added. Samples were kept at −20 °C for 30 min. The fixative was changed three times. After the final centrifugation, the supernatant was replaced by a freshly prepared fixative (1–2 mL) and such prepared cell suspensions were transferred to plastic tubes and stored at

−20 °C for the further use. Microscope slides were prepared by placing one drop of the cell suspension from a height of 20–30 cm on a slide and left to dry.

2.4. Interphase Quantitative Fluorescence In Situ Hybridization (Q-FISH)

Microscope slides with rainbow trout cells were prepared the day before Q-FISH procedure and kept at the room temperature. Telomeric DNA repeats were detected by FISH, using a Telomere PNA (peptide nucleic acid) FISH Kit/FITC (DAKO, Glostrup, Denmark) according to the manufacturer's protocol. Chromosomal DNA was denatured at 85 °C for 5 min under the cover slip in the presence of the PNA probe. During hybridization, microscope slides were left in the dark at room temperature for 1 h. Fifteen minutes before microscopic analysis cells were counterstained with DAPI in Vectashield antifade solution (Vector Laboratories, Burlingame, CA, USA). FISH was performed on two slides from each studied specimen.

Each slide was scanned with the ASI system, HiFISH-SpotScan module (Applied Spectral Imaging, Yokne'am Illit, Israel) with a dedicated 5 M CMOS camera connected to a fluorescent BX53 Olympus microscope. Ten (10) fields of view (frames) were captured under 100× magnification with DAPI and FITC filters in the multiple focal planes to ensure all signals will be in focus for the correct analysis. SpotScan automatically detected all cells in the scanned region and the telomere fluorescent signals within them. Quantitative analysis of the telomere fluorescence intensity was performed on the classified cells. Average telomere fluorescence intensities and standard deviations were automatically reported per 100–200 cells from the scanned regions. Detailed data concerning particular frames are available on request due to privacy/ethical restrictions. The optical and software setup was kept identical along the scan of the slide and between slides. Note that the intensity values were given in the arbitrary units, as acceptable in such cases. Any conclusion was derived by comparison between paralleled experiments.

2.5. Statistical Analysis

Data were analyzed using R software version 3.5.3 (11 March 2019). Normal distribution was tested by Shapiro–Wilk test. Correlations between telomere length-related fluorescence and fish body weight (1) and length (2) were assessed using Pearson or Spearman test, according to the data distribution.

Since age, ploidy, body size (length and weight) and interactions between these variables may affect telomere length, results were analyzed using analysis of covariance (ANCOVA) and general linear model (GLM) was provided. As body length and weight are correlated, two separate ANCOVA were performed. Afterward, ANOVA post hoc Tukey's test was applied.

3. Results

3.1. Body Weight and Length and Gonadal Development

Body length and weight of the examined specimens increased with age, what confirmed that rainbow trout grow continuously throughout life. Diploid embryos, larvae, juveniles and subadults exhibited significantly lower body length than triploids while adults, irrespective of the ploidy, showed similar length ($p < 0.05$). In turn, triploid larvae, juveniles and two-year-old trout were substantially heavier than their diploid counterparts (Table 1). Weight of embryos and adults from diploid and triploid stocks did not differ substantially ($p > 0.05$).

Ovaries were macroscopically observed only in the diploid juveniles and subadults. In diploid adult females, oocytes were found in the body cavity. In the triploid individuals neither oocytes nor properly developed ovaries were observed what confirmed sterility of these fish.

Table 1. Parameters of rainbow trout body weight and length. (*) indicate statistically significant difference between diploid and triploid fish at the same stage of development.

Stage of Development	Ploidy	Length (cm)		Weight (g)	
		Mean	±SD	Mean	±SD
Embryos	2n	2.08 *	0.07	0.12	0.009
	3n	2.19 *	0.10	0.12	0.010
Larvae	2n	2.43 *	3.14	0.12 *	0.017
	3n	3.08 *	0.07	0.21 *	0.021
Juveniles (one year old)	2n	14.30 *	1.30	26.12 *	5.01
	3n	17.70 *	1.31	60.28 *	11.80
Subadults (two years old)	2n	22.72 *	2.49	126.83 *	47.10
	3n	26.48 *	1.55	204.00 *	46.08
Adults (three years old)	2n	32.26	1.80	425.20	109.20
	3n	35.00	2.37	442.40	151.76

3.2. Dynamics of Telomere Length in Rainbow Trout

Q-FISH on the rainbow trout interphase diploid and triploid cells was successfully applied to follow changes of the telomere length with age and body growth (Figure 1). Mean values of the telomere length-related intensity of fluorescence for individuals from each age category and ploidy are provided (Table S1). In the diploid rainbow trout embryos, larvae and juveniles, telomere length-related fluorescence presented in the fluorescent arbitrary units ×10 equaled 24.94 ± 6.07 (mean ± SD), 26.61 ± 4.91 and 21.73 ± 5.06, respectively. Substantially shortened telomere length was observed only in the subadults (12.57 ± 2.07) when compared to telomeres of fish from the earlier stages of development (Figure 2a). Q-FISH analysis of adult diploid rainbow trout showed an unexpected increase of the telomere length 18.13 ± 4.15 (Figure 2a).

In triploids, Q-FISH analysis exhibited similar telomere length in embryos (30.71 ± 7.13) and larvae (29.38 ± 6.1). Then telomere length was decreasing to reach 18.53 ± 2.99 evidenced in the juveniles and 11.38 ± 1.97 reported in subadult individuals (Figure 2b). When compared to subadult fish, adult triploids showed increased telomeres (18.13 ± 4.15) that reached similar length to that observed in juveniles (Figure 2b).

Results of ANCOVA showed that age together with interaction between ploidy and age affected telomere length ($p < 0.05$). However, ploidy itself did not show correlation with the telomere dynamics. Post hoc analysis exhibited statistically significant difference in the telomere length between subadults and embryos, subadults and larvae, subadults and adult fish ($p < 0.05$).

3.3. Correlation between Body Weight and Length and Telomere Length-Related Fluorescence

Statistical analysis showed substantial correlation between telomere length and body length ($r^2 = 0.69$, $p > 0.05$) and between telomere length and body weight ($r^2 = 0.52$, $p > 0.05$) in the triploid rainbow trout (Figures 3 and 4). For diploid fish, these correlations were lower and not statistically significant ($r^2 = 0.07$, $r^2 = 0.34$, respectively) (Figures 3 and 4). Considering age categories, the biggest correlation (but not statistically significant) between telomere length and body size and weight was found in one-year-old diploids ($r^2 = 0.79$, $r^2 = 84$).

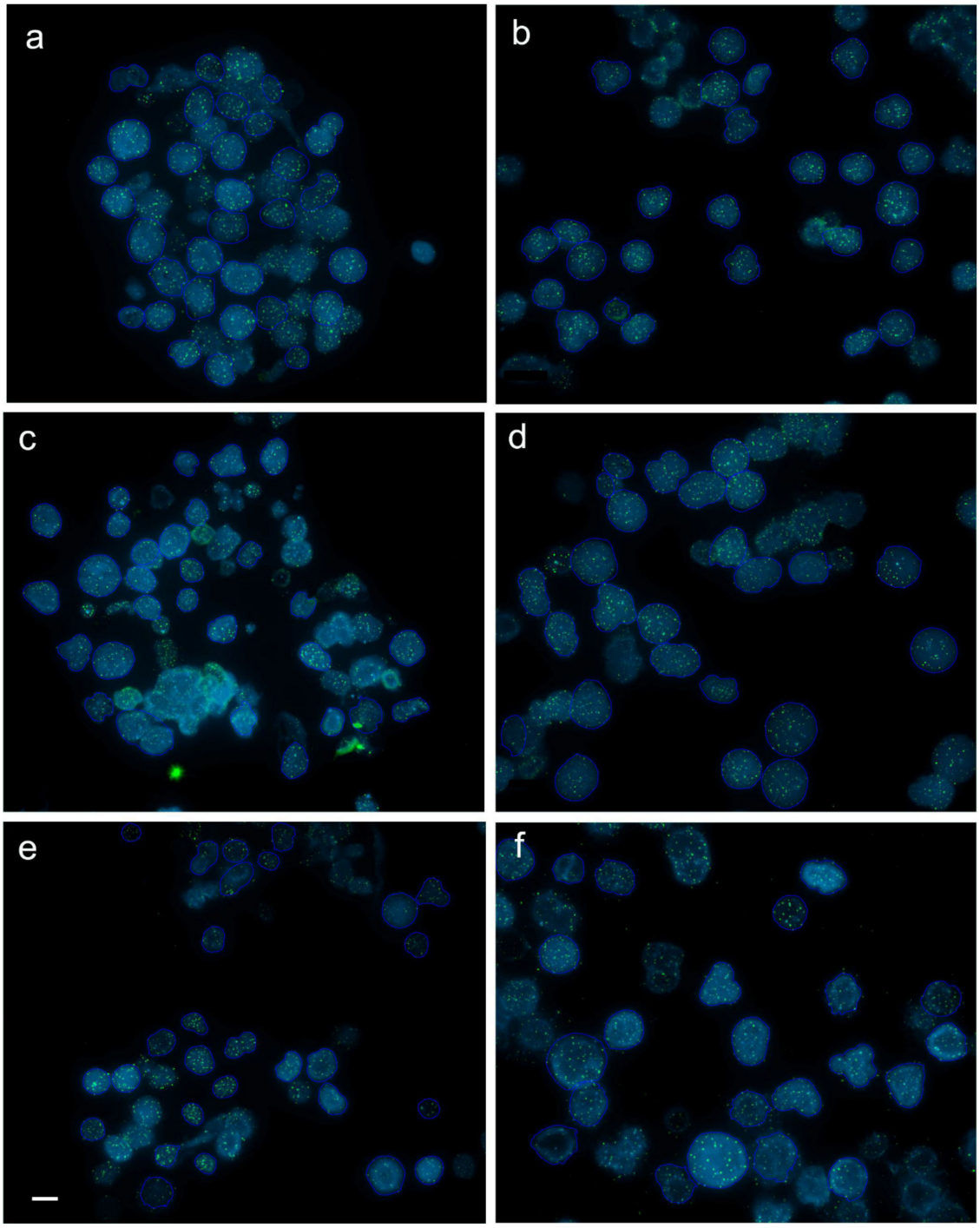

Figure 1. Captured images (frames) with interphase cells from juvenile (**a**,**b**), subadult (**c**,**d**) and adult (**e**,**f**) diploid (**a**,**c**,**e**) and triploid (**b**,**d**,**f**) rainbow trout after fluorescence in situ hybridization (FISH) with PNA (peptide nucleic acid) telomere probe. Average intensity of telomere fluorescence (×10) for cell from the captured frames in juveniles, subadults and adults equaled 18.8 ± 3.3 (**a**) and 18.3 ± 1.8 (**b**), 13.7 ± 1.4 (**c**) and 13.1 ± 1.4 (**d**), 26.3 ± 5.5 (**e**) and 19.3 ± 5.6 (**f**), respectively. Scale bar = 10 μm.

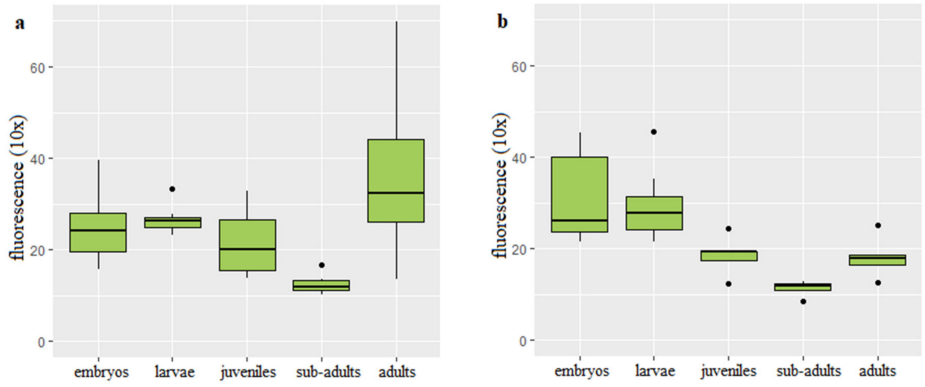

Figure 2. Telomere length-related fluorescence reported in diploid (**a**) and triploid (**b**) rainbow trout at different stages of development.

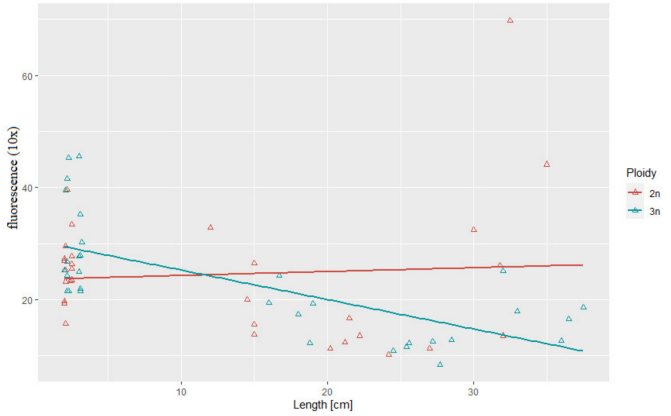

Figure 3. Relationship between telomere length-related fluorescence and body length in the examined triploid ($r^2 = 0.69$, $p > 0.05$) and diploid ($r^2 = 0.07$) rainbow trout.

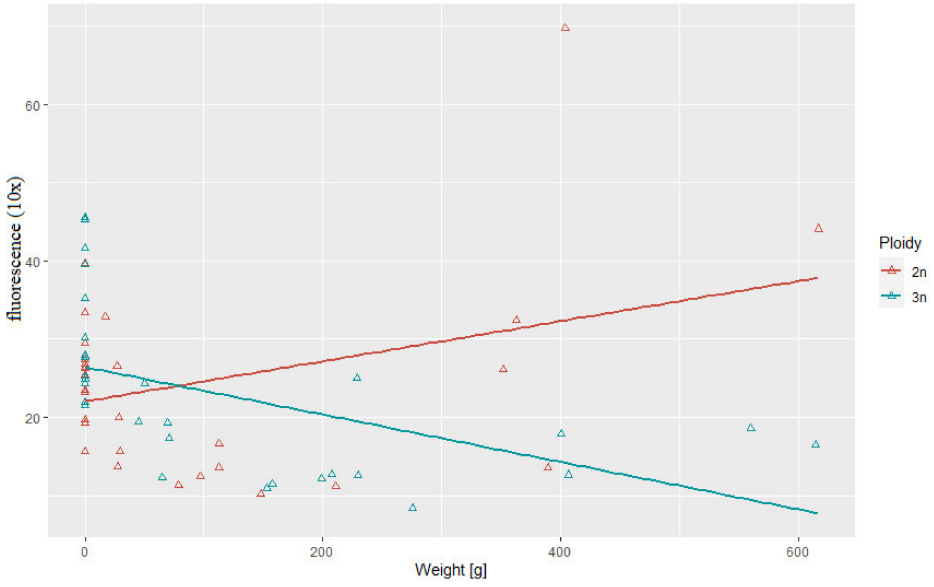

Figure 4. Relationship between telomere length-related fluorescence and body weight observed in examined triploid ($r^2 = 0.52$, $p > 0.05$) and diploid ($r^2 = 0.34$) rainbow trout.

4. Discussion

Although chromosomal distribution of telomeric repeats has been studied in many salmonids [34–37], there are only few published data concerning dynamics of telomeric DNA in salmon and trout [38–40]. In this research we reported age-related decrease and increase of telomere length in the diploid and triploid rainbow trout. Lengths of telomeres in the rainbow trout embryos, larvae and juveniles were similar. Significantly shortened telomeres were noticed in the subadult individuals during the second year of life (Figure 1). Unexpectedly, after substantial telomere loss observed in the two-year-old rainbow trout, telomere length in the three-year-old diploids and triploids was increased to reach the size observed in the one-year-old juveniles (Figure 1).

As similar patterns of telomere-shortening, and lengthening were observed in the fertile and sterile fish it may be presumed that processes related to reproduction do not affect dynamics of telomeres in the rainbow trout. Nevertheless, maybe the same dynamics of the telomere length should not be surprising as except issues related to sterility such as reduced gonads and decreased levels of gonadotropins, sex steroids and vitellogenin, triploid salmonid are frequently observed to be morphologically and physiologically similar to the diploid individuals [25,26]. Although, triploid fish should grow faster than diploids due to the cell size, increased heterozygosity and sterility, most observations confirmed that triploid salmonids during juvenile stage usually show equal or even worse growth than diploids and only after maturation the growth is enhanced in the triploid individuals [25]. Till juvenile stage, triploid and diploid rainbow trout have been found to have comparable energy expenditure, oxidation of glucose and amino acids or excretion rate [26,41–43]. In chinook salmon, no differences in the gene expression was observed between ploidies even though triploid individuals were characterized by the lower growth rate [44]. Some studies evidenced similar reaction for the environmental conditions in fish with different ploidy [45]. Triploid and diploid Atlantic salmon have similar physiological stress response including comparable expression of the oxidative stress genes [46].

Decline in telomere length observed in rainbow trout in the second year of life may be consequence of a fast growth that is specific for the premature phase in fish. Results of studies performed on both endotherms and ectotherms including fish evidenced that pace of telomere attrition is the highest during early life stages characterized by the rapid growth when cell division rate is increased [21]. Experimentally enhanced growth rate observed in the transgenic salmon was followed by much higher telomere shortening when compared to their non-manipulated siblings [40]. Interestingly, only in triploid rainbow trout correlation between telomere attrition and body size (length and weight) was observed. However, triploid specimens in this experiment were bigger than their diploid counterparts at every developmental stage from hatching till subadult stage what could have affected dynamics of the telomere change (Table 1). Results of studies concerning relationship between telomere length and animal body size are inconsistent. In humans, wild house sparrows and American Alligator increased body size is associated with the reduced telomere length [13,47]. On the contrary, no significant correlation between telomere loss rate and change in the body mass was observed in long-lived barnacle goose [48] and red-sided garter snakes [49].

Telomere shortening observed in the subadults has not been compensated by telomerase despite its high activity confirmed in rainbow trout various organs including kidney irrespective of the fish age [8]. This may suggest that level of telomerase was not sufficient to prevent growth-related telomere attrition. However, after significant telomere loss observed in the two-year-old rainbow trout, telomere length-related fluorescence in the three-year-old diploids and triploids was increased to reach the size observed in the one-year-old juveniles (Figure 1). It is not a first case when an increase of the telomere length occurs after age-related shortening. In medaka, telomeres shorten during rapid growth observed at the early stages of development, lengthen during adolescence when the growth rate slows down and shorten again what is correlated with the reduced growth [50]. In turn, in zebrafish, the telomere length increases earlier, from larvae to the adult fish then stabilizes and eventually declines substantially [20]. The mechanism responsible for the telomere elongation in the adult rainbow trout is unclear though, it can be considered that telomerase that was not able to maintain length of the

telomeres during period of the rapid growth enabled not only compensation for the telomere attrition, but increase of the telomere length in the three-year-old adults which growth rate slows down a little bit (Table 1). Moreover, it is not excluded that other cellular mechanisms such as reciprocal recombination and transposition of the chromosomal terminal elements [51] may have been also utilized for the rainbow trout telomere elongation in the third year of life. In cells with knocked down telomerase reverse transcriptase (TERT) subunit, telomeres shortening was limited by independent of telomerase mechanism termed alternative lengthening of telomeres (ALT) [52].

5. Conclusions

Q-FISH analysis was used to study dynamics of telomere length in diploid and triploid rainbow trout with aging. Behavior of telomeres in diploid and triploid trout showed similar patterns that included decline of telomeres observed in the two-year-old individuals followed by increase of telomeres reported in three-year-old adults. The reduction of telomere length observed to occur within first two years after hatching was paralleled with the period of the fast growth. Similar pattern of the telomere dynamics observed in the diploid and sterile triploid indicated processes related to reproduction did not affect telomere dynamics in rainbow trout.

Supplementary Materials: The following are available online at http://www.mdpi.com/2073-4425/11/7/786/s1, Table S1: Telomere length related fluorescence of all examined diploid and triploid rainbow trout specimens (averages and standard deviations).

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Table S1 Telomere length related fluorescence of all examined diploid and triploid rainbow trout specimens

symbol of examined fish	Stage of development	Ploidy	Fluorescence (auf)	sd
1de	Embryo	2N	25,25	6,8
2de	Embryo	2N	29,5	9,55
3de	Embryo	2N	19,25	3,2
4de	Embryo	2N	19,65	5,65
5de	Embryo	2N	27,35	7,45
6de	Embryo	2N	23,2	3,2
7de	Embryo	2N	15,7	1,9
8de	Embryo	2N	39,65	10,8
1te	Embryo	3N	26,8	6,7
2te	Embryo	3N	45,25	11,5
3te	Embryo	3N	21,5	3,5
4te	Embryo	3N	41,6	11
5te	Embryo	3N	24,3	5,85
6te	Embryo	3N	39,5	9
7te	Embryo	3N	21,5	4,35
8te	Embryo	3N	25,25	5,1
1ld	Larva	2N	25,45	5,1
2ld	Larva	2N	26,4	5,75
3ld	Larva	2N	27,75	6,3
4ld	Larva	2N	26,85	3,7
5ld	Larva	2N	23,25	3,15
6ld	Larva	2N	23,5	4,85
7ld	Larva	2N	33,35	6,1
8ld	Larva	2N	26,35	4,35
1lt	Larva	3N	21,95	5,3
2lt	Larva	3N	21,5	5,6
3lt	Larva	3N	35,25	7,3
4lt	Larva	3N	30,15	6,15
5lt	Larva	3N	24,95	5,4
6lt	Larva	3N	27,9	6,2
7lt	Larva	3N	27,75	3,85
8lt	Larva	3N	45,6	9
1dj	1 year old	2N	32,85	9,25
2dj	1 year old	2N	13,7	2,5

3dj	1 year old	2N	26,5	7,25
4dj	1 year old	2N	15,6	2,15
5dj	1 year old	2N	20	4,15
1tj	1 year old	3N	24,3	4,55
2tj	1 year old	3N	17,35	3,1
3tj	1 year old	3N	19,35	2,75
4tj	1 year old	3N	19,4	2,8
5tj	1 year old	3N	12,25	1,75
1ds	2 years old	2N	16,7	2,5
2ds	2 years old	2N	11,25	1,3
3ds	2 years old	2N	12,4	1,4
4ds	2 years old	2N	11,3	1,65
5ds	2 years old	2N	13,55	2,15
6ds	2 years old	2N	10,2	2,3
1ts	2 years old	3N	11,5	3
2ts	2 years old	3N	8,4	0,95
3ts	2 years old	3N	10,85	2,05
4ts	2 years old	3N	12,55	1,55
5ts	2 years old	3N	12,75	2,3
6ts	2 years old	3N	12,2	1,95
1da	3 years old	2N	44,1	15,35
2da	3 years old	2N	32,4	6,75
3da	3 years old	2N	69,75	7,7

4da	3 years old	2N	26,1	5,1
5da	3 years old	2N	13,55	1,65
1ta	3 years old	3N	12,65	1,95
2ta	3 years old	3N	25,05	7,5
3ta	3 years old	3N	18,55	4,2
4ta	3 years old	3N	17,85	5,45
5ta	3 years old	3N	16,55	1,65

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ANIMAL GENETICS • ORIGINAL PAPER

Telomere length variation does not correspond with the growth disturbances in the rainbow trout (*Oncorhynchus mykiss*)

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Abstract
Somatic growth is considered to affect pace of the telomere attrition in vertebrates. As normally developed and dwarf fish differ in the body size we have decided to compare telomere length in the rainbow trout (*Oncorhynchus mykiss*) with normal growth and with growth reduced due to the dwarf condition. Examined 1-year-old fish with normal and dwarf appearance were siblings originated from androgenetic fully homozygous doubled haploid (DH) line of rainbow trout. Particular dwarf individuals had body deformities such as humpback, kyphosis, and lordosis. Somatic cells of examined rainbow trout had an average telomere length between 17 and 20 kb, comparable in females and males. Dwarf rainbow trout exhibited significantly lower body length and weight than their normally developed siblings even though no differences in the telomere length were found between these fishes. Statistical analysis did not exhibit any correlation between body size and the telomere length. Equal length of telomeres observed in the studied normal and dwarf rainbow trout suggests morphological and physiological differences in fish with different growth rates do not affect dynamics of telomeric DNA. Or any variation in the telomere length might have been levelled by telomerase that in rainbow trout is active in all tissues irrespective of the individual developmental stage.

Keywords Body size · Dwarfism · Growth · Telomerase · Telomeres

Introduction

Telomeres, the nucleoprotein complexes located at the ends of linear eukaryotic chromosomes that assure their complete replication, protect their ends from DNA degradation, DNA repair mechanism, and fusions with other chromosomes (De Lange 2002; Chan and Blacburn 2004; Bolzán and Bianchi, 2006). Telomeres shorten with every cell division as the DNA polymerase is not able to replicate ends of the linear chromosomes ("end replication problem") (Olovnikov 1973). Furthermore, shortening of telomeres is accelerated by the oxidative stress associated with increased production of reactive oxygen species (ROS) (Von Zglinicki 2002). ROS are mainly produced in mitochondria during process of oxidative metabolism, thus somatic growth as energetically costly process is considered to affect dynamics of telomere attrition (Von Zglinicki 2002). Somatic growth may lead to decrease telomere length by two main paths; by increased cell division rate that is indispensable for growing of the organisms (1) or/and by increased energy expenditure and hence increased oxidative stress/ROS generation leading to acceleration of DNA damage (Monaghan and Ozanne 2018). Shortening of telomeres can be slowed by telomerase that catalytic component, TERT (telomerase reverse transcriptase), adds telomeric DNA repeats using as a template an integral RNA element (TR, telomerase RNA). Telomerase activity in humans is restricted to germ line cells,

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stem cells, and tumors (Chan and Blackburn 2004). In turn, in fish, activity of telomerase has been confirmed in many organs irrespective of size and age of examined specimens [Klapper et al. 1998; Elmore et al. 2008; Lund et al. 2009].

Scoliosis, lordosis, or kyphosis are quite frequently observed in fish from the wild stocks as well as from the aquaculture (Boglione et al. 2013). Due to the increased metabolic costs, somatic growth in individuals with spinal deformities may be retarded what usually results in dwarfism (Witten et al. 2009; McMenamin et al. 2013; Shao et al. 2019). In experiments concerning production of rainbow trout (*Oncorhynchus mykiss*) androgenetic and gynogenetic doubled haploids (DHs), dwarf individuals with spinal deformities have been frequently observed (Ocalewicz et al. 2010, 2013, among others). Growth retardation makes dwarf fish interesting objects to study dynamics of the telomeric DNA. On the one hand, dwarf fish may have decreased ratio of cell divisions; on the other hand, such fish presumably show increased metabolic costs (Shao et al. 2019) what may affect pace of the telomere shortening. Therefore, the main goal of the present research was to examine changes of the telomere length in the dwarf and normal rainbow trout.

Materials and method

This study was carried out in strict accordance with the recommendations in the Polish ACT of 21 January 2005 of Animal Experiments (Dz. U. of. 2005 No 33, item 289). The protocol was approved by the Local Ethical Committee for the Experiments on Animals in Bydgoszcz.

Fish origin and breeding conditions

Androgenetic doubled haploids (DHs) and their normal heterozygous siblings studied in this research were produced and maintained using gamete donors from the spring spawning broodstock of rainbow trout from Rutki strain reared in the Department of Salmonid Research (DSR), Inland Fisheries Institute in Olsztyn (IFI), Rutki, Poland (Polonis et al. 2021). Shortly, batches of eggs were divided in to two groups; one designated for irradiation and induction of androgenesis and the second batch to serve as control (normal fertilization). Eggs were irradiated with 350 Gy of X-rays (6 Gy/min) using TrueBeam linear accelerator (Varian Medical Systems, Palo Alto, CA, USA) and two opposed field technique (175 Gy from each field). Eggs from the irradiated and non-irradiated batches were inseminated with 0.15 ml of milt for about 150 g of eggs in the presence of the sperm activating medium (SAM). Eggs were rinsed with the hatchery water 5 min after activation. After 350 min since insemination fertilized and irradiated eggs were exposed to the high hydrostatic pressure (HHP) shock (9500 PSI for 3 min) using TRC-APV electric/hydraulic apparatus (TRC Hydraulics Inc. Dieppe, Canada), this process is intended to duplicate paternal set of chromosomes and to create androgenetic doubled haploids (DHs). Control groups were provided by fertilization of the non-irradiated eggs from each female. Both androgenetic and control groups were incubated in three replicates at 10 °C under routine hatchery conditions.

Fish sampling and measurements

One-year-old dwarf androgenetic individuals ($n = 7$), normally developed androgenetic specimens ($n = 7$) and their normally developed siblings from control group ($n = 7$) were sampled and examined. The sample size was determined to meet requirements for the statistical analysis and it was based on the previous studies using Q-FISH performed in zebrafish (Anchelin et al. 2011) and rainbow trout (Panasiak et al. 2020). All rainbow trout were maintained under the same husbandry and environmental conditions. Sampled fish were sacrificed by an overdose of propiscin (Etomidatum, IFI) (Inland Fisheries Institute, Olsztyn, Poland), weighed and their length measured (Table 1).

Preparation of interphase spreads

Somatic cells of all sampled fish were prepared from the head kidney tissue. This particular tissue was chosen, because it is commonly used in the fish cytogenetic analysis for preparation of the interphase spreads (Panasiak et al. 2020). Pieces of the head kidneys were homogenized in 5 ml of KCl (0.075 M) and left for 40 min at room temperature. Afterward, ice cold fixative (methanol: acetic acid, 3:1) was added and the tubes were centrifuged at 1000 rpm for 10 min. Supernatant was removed, fresh fixative was added, and samples were centrifuged again. This step was repeated three times. After last centrifugation, supernatant was replaced by a fresh fixative and cell suspension was transferred to the plastic tubes and stored at − 20 °C.

Preparation of cell line L5178Y-R

Murine DBA/2 lymphoma cell line L178Y-R (European Collection of Authenticated Cell Cultures via Merck KGaA, Darmstadt, Germany; LOT06/F/004) with known telomere length (79.7 kb) has been used in the present research as a reference for the Q-FISH analysis of the rainbow trout cells and to assess length of the trout telomeric DNA The culture was carried using RPMI1640 medium (Carl Roth GmbH) supplemented with 10% fetal bovine serum (Merck KGaA) and penicillin–streptomycin solution (50u and 0.05 mg per 1 ml of medium respectively; Merck KGaA) and maintained between 3×10^4 to 7×10^5 cells per ml. Incubation was carried out at 37 °C with 5% CO_2 and continuous shaking

Table 1 Sex, body size, telomere length-related fluorescence (TLF) and estimated telomere length of rainbow trout: androgenetic dwarfs ($_{d}$DH), normal androgenotes ($_{n}$DH), and their siblings from control group (C)

Number of individual	Sex	Body weight [g]	Body length [cm]	TLF	Estimated telomere length [kb]
$_{d}DH_1$	M	23.56	12	11.95 ± 2.45	16.12
$_{d}DH_2$	F	45.05	15.9	12.8 ± 5.45	17.27
$_{d}DH_3$	F	36.07	13.7	15.85 ± 3.7	21.39
$_{d}DH_4$	M	78.8	16.5	18.4 ± 6	24.83
$_{d}DH_5$	M	32	15	12.9 ± 4.1	17.41
$_{d}DH_6$	F	44.1	14.5	11.55 ± 2.1	15.58
$_{d}DH_7$	M	36.7	14.5	10 ± 1.4	13.49
$_{n}DH_1$	F	77.8	18.3	13.05 ± 4.1	17.61
$_{n}DH_2$	M	137.6	23	13.15 ± 3	17.74
$_{n}DH_3$	F	104.3	21.2	11.25 ± 1.95	15.18
$_{n}DH_4$	M	143	22	10.6 ± 2.95	14.30
$_{n}DH_5$	F	148.7	23.5	11.95 ± 1.95	16.12
$_{n}DH_6$	F	64.5	17.6	13.3 ± 2.25	17.94
$_{n}DH_7$	F	101.9	21.2	14.35 ± 3.25	19.36
C_1	M	97.9	21.4	13.15 ± 2.55	17.74
C_2	F	93.8	20.8	13.55 ± 3.75	18.28
C_3	M	185.3	24	20.85 ± 4.25	28.13
C_4	F	86.1	20.5	12.4 ± 2	16.73
C_5	M	158.8	23	20.25 ± 5.5	27.32
C_6	F	139.1	23.5	15.25 ± 3.25	20.58
C_7	F	154.9	23.5	11.55 ± 3.25	15.58

at 90 rpm (New Brunswick™ S41i Incubator, Eppendorf AG) in 125-ml single use, vented, PETG Erlenmeyer flasks (Thermo Fisher Scientific Inc.). About 72 h prior to preparation of the interphase spreads, the cells were seeded to 50 ml of fresh culture medium at 10^6 cells per 1 ml. Incubation was conducted as mentioned above. After 72 h, the whole volume of the culture was centrifuged at 1000 rpm. Interphase spreads were prepared in the same way as the head kidney cells, despite the hypotonization step which lasted 45 min.

Interphase quantitative fluorescence in situ hybridization (Q-FISH)

Telomeric DNA repeats were detected using a Telomere PNA (peptide nucleic acid) FISH Kit/FITC (DAKO, Glostrup, Denmark) according to the manufacturer's protocol. Microscopic slides with fish cells and DBA/2 lymphoma cells were prepared the day before PNA-FISH experiment and kept at the room temperature. Q-FISH analysis has been performed using the method described in details in (Panasiak et al. 2020). Shortly, after the PNA-FISH slides were scanned with the ASI system, HiFISH-SpotScan module (Applied Spectral Imaging, Yokne'am Illit, Israel) with a dedicated 5 M CMOS camera connected to a fluorescent BX53 Olympus microscope. Ten fields of view (frames) were captured under 100 × magnification with DAPI and FITC filters in the multiple focal planes to ensure all signals will be in focus for the correct analysis. All cells and the telomere fluorescent signals in the scanned region were automatically detected by HiFISH-SpotScan software. Quantitative analysis of the telomere fluorescence intensity was performed only on the classified cells. Average telomere fluorescence intensities and standard deviations were reported per 100–200 cells from the scanned regions (Supplementary File 1). The intensity values were given in the arbitrary units, as acceptable in such cases.

Statistical analysis

Provided data were analyzed using R software version 1.3.959 (Supplementary File 2). Normal distribution was tested by Shapiro–Wilk test and Levene's test was applied to check homogeneity of variances between analyzed groups of rainbow trout. Correlations between telomere length-related fluorescence and fish body length and weight were assessed using Pearson or Spearman test, according to the data distribution. ANOVA was used to determine if there were any significant differences between means of the telomere length-related fluorescence and body size of all the examined groups of fish. Due to lack of variance homogeneity, difference in the body weight between dwarf individuals and fish from control group was estimated using Kruskall-Wallis.

T-test was applied to determine if there is any significant difference in the telomere length-related fluorescence between normal rainbow trout males (XY) and females (XX) and between androgenetic males (YY) and females (XX).

Results

The mean values of body weight/length of normally developed androgenotes, androgenetic dwarfs`, and heterozygous trout from the control group equaled 111.11 g/20.97 cm, 42.33 g/14.59 cm, and 130.84 g/ 22.39 cm, respectively (Fig. 1, Table 1). Dwarf androgenetic rainbow trout exhibited significantly lower body length and weight when compared to androgenetic fish with normal appearance and trout from the control group ($p < 0.05$). Particular dwarf androgenetic fish had visible morphological changes for example humpback, kyphosis, and lordosis. There were no significant differences in the body size between normally developed androgenetic trout and fish from the control group.

Telomere length-related fluorescence (TLF) (expressed in the fluorescent arbitrary units × 10) in the normally developed androgenetic rainbow trout, androgenetic dwarfs, and control rainbow trout equaled 12.52 ± 2.78, 13.42 ± 3.60, and 15.29 ± 3.51 (mean ± SD), respectively (Supplementary File 1, Fig. 1). For L178Y-R cells, TLF was 59.07 ± 6.93.

Results of statistical analysis showed that there were no statistically significant differences between telomere length-related fluorescence in rainbow trout from different examined groups. Statistical analysis did not show any correlation between body weight or length and the telomere length-related fluorescence in the examined fish (Fig. 2). Moreover, no significant differences in the telomere length between females and males were found among androgenetic fish and among their heterozygous siblings from the control group.

Discussion

A slower growth rate is observed in the large species when compared to the small bodied species; however the opposite is observed within the species where large specimens grow faster than the smaller ones (Monaghan and Ozanne 2018). Due to the increased cell divisions, higher metabolic rate, and related increased generation of ROS, we may expect that fast growing individuals exhibit shorter telomeres. On the other hand, faster growth may be related to better conditions of life and smaller oxidative stress that in turn should slow down pace of the telomere attrition. As normal and dwarf fish differ in the body size and the growth rate, we also expected to see differences in the telomere length between individuals with normal and retarded growth. Average telomere length-related fluorescence (TLF) in 1-year-old rainbow trout was 15.21 (fluorescent arbitrary units × 10) and androgenotes and their heterozygous siblings from the control group had telomeres of similar size. In comparison of TLF in rainbow trout cells and in L5178Y-R cells with known telomere length indicates, that average telomere length of the examined rainbow trout may equal about 20 kb what is in agreement with previously published estimation provided in the course of other than Q-FISH method for the telomere length measurement, namely terminal restriction fragment (TRF) analysis by Southern blot analysis (Lejnine et al. 1995). In spite of significant differences in the body length and weight observed between androgenetic DH rainbow trout with normal growth and those with the retarded growth, no significant differences in the telomere length

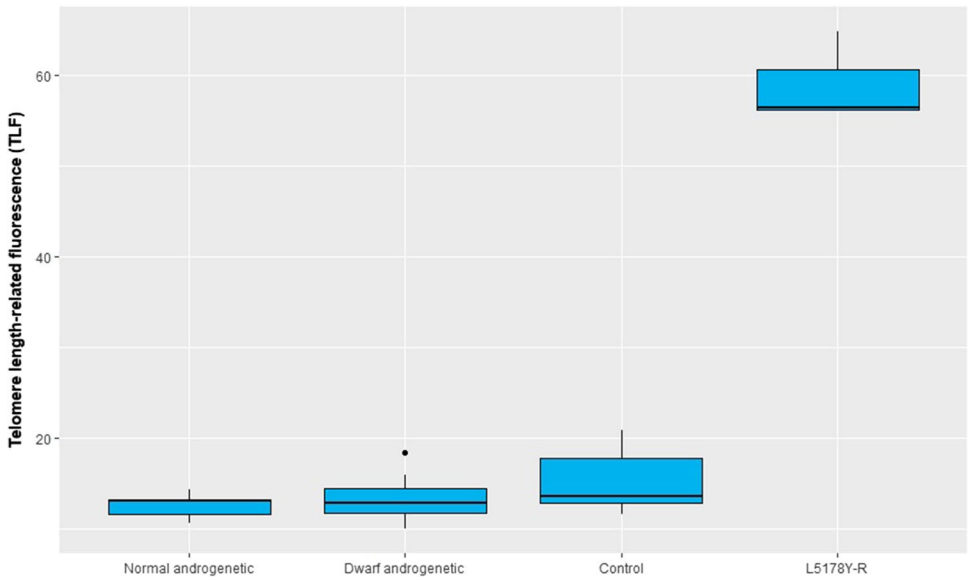

Fig. 1 Values of telomere length-related fluorescence (expressed in the fluorescent arbitrary units × 10) in cells from examined rainbow trout and L5178Y-R cells

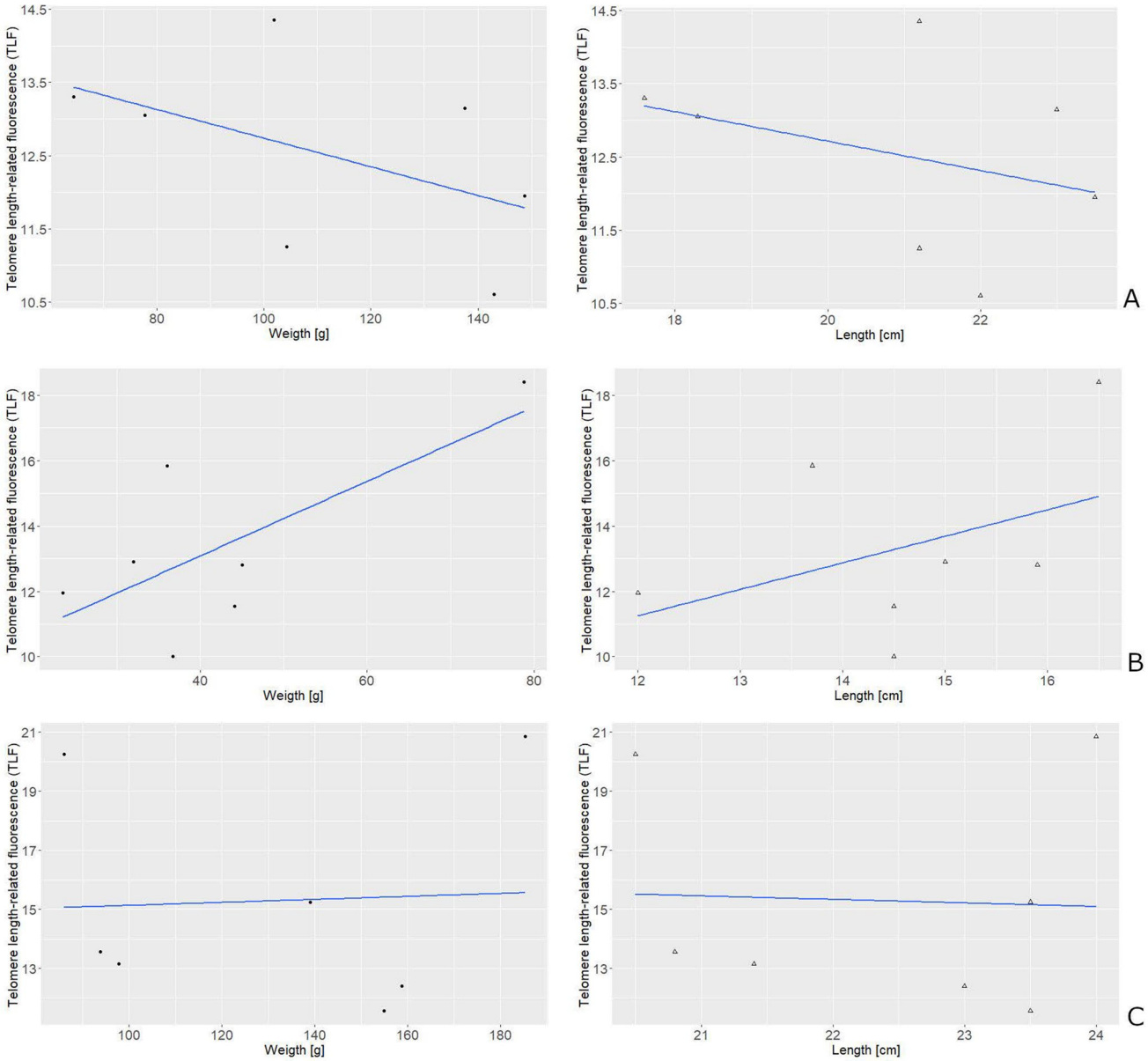

Fig. 2 Relationship between telomere length-related fluorescence (TLF) (×10) and body weight and length in the androgenetic rainbow trout with normal growth rate ($r=0.495$; $p=0.259$ and $r=0.344$; $p=0.450$, respectively) (**A**), in the androgenetic rainbow trout with growth retardation ($r=0.708$; $p=0.075$ and $r=0.421$; $p=0.347$, respectively) (**B**) and in the rainbow trout from control group ($r=0.051$; $p=0.913$ and $r=0$ .458; $p=0.922$, respectively (**C**)

were found between these fishes (Fig. 2). Provided results have proven that retarded growth observed in the dwarf rainbow trout is not followed by changes in the dynamics of the telomeric DNA. Equal length of telomeres observed in the trout siblings with normal and abnormal body size suggests that any differences in the number and pace of cell divisions, metabolic costs, and thereby oxidative stress in rainbow trout with different growth rate do not affect their telomere length. On the other hand, it is not excluded that any differences in the telomere length resulted from variation in the somatic growth rate and body size between dwarfs and normally developed trout examined here are levelled by telomerase that in rainbow trout is active in all tissues irrespective of individual developmental stage (Klapper et al. 1998).

Except for no differences in the telomere length between normal and dwarf rainbow trout, issue that draws attention was an inter-individual variation of the telomere length in the examined specimens (Table 1). Telomere length is a

complex heritable trait and the inter-individual variation in the telomere length appears during early developmental stages as the interaction between genetic and predominant environmental factors including oxidative stress, inflammation, or physiological stress (Srinivas et al. 2020). On the other hand, it is not excluded that such variation was related to the tissue that had been used for cell preparation. Fish head kidney is a hemopoietic organ forming blood elements where processes of hemopoiesis including erythropoiesis, granulopoiesis, monopoiesis, thrombopoiesis, and lymphoplasmopoiesis occurs. The head kidney contains varied types of cells at different stages of maturity (Abdel-Aziz et al. 2010) what may affect telomere length in these cells. Similar inter-individual variation in the telomere length has been confirmed in killifish (*Fundulus heteroclitus*), dogfish shark (*Squalus acanthias*), little skate (*Raja erinacea*), American eel (*Anguilla rostrata*), or zebrafish (Elmore et al. 2008; Lund et al. 2009). Highly heterogeneous telomeres in terms of length have been observed in the liver cells of Japanese medaka (*Oryzias latipes*) (6 to 12 kb) and *O. melastigma* (0.5 to 12 kb) (Au et al. 2009).

In many vertebrate species, females have longer telomeres and live longer than males (Barrett and Richardson 2011). Such pattern of the telomere length and lifespan have been also described in medaka (Gopalakrishnan et al. 2013) and *Nothobranchius furzeri* (Reichard et al. 2020) while in the common carp (*Cyprinus carpio*) (Izzo et al. 2014) and rainbow trout examined here females and males exhibited comparable length of telomeric DNA. Different patterns of telomere dynamics reported between sexes in several fish species may mirror interspecies differences related to the lifespan and sex differentiation. On the other hand, it is not excluded that in the rainbow trout any differences in the telomere length between females and males might appear only in the sexually matured and adult individuals. As oestrogens has been shown to stimulate telomerase activity (Viña et al. 2005), it may be presumed that high level of this steroid observed in females but not in males is at least in part responsible for the lower rates of telomere attrition in females. Studied in the present research, one-year-old rainbow trout were not sexually matured thus inter sex differences in levels of oestrogens might have been too low to affect telomere length in the sex specific manner. To answer these doubts, fully matured rainbow trout females and males should be examined and the sample size needs to be larger.

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Author contribution Conceptualization and experimental design (KO, LP), induction of androgenesis (KO, MP), fish measurement and sampling (KO and LP), cell culture and harvest (KS, LP), Q-FISH and statistical analysis (LP), writing and editing (KO, LP, KS), and funding acquisition and supervision (KO). All authors have read and agreed to the published version of the manuscript.

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Declarations

Consent to participate Not applicable.

Consent for publication Not applicable.

Conflict of interest The authors declare no competing interests.

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Supplementary File 1. Results of Q-FISH analysis showing telomere length-related fluorescence (expressed in the fluorescent arbitrary units × 10) in cells from examined rainbow trout.

Supplementary File 2. R-script for conducting analysis related to rainbow trout telomere length and body size. Available at the link: https://link.springer.com/article/10.1007/s13353-021-00669-6

	TLF	SD		TLF	SD		TLF	SD		TLF	SD
ANDRO1	11,7	2,5	DWARF1	11,9	2,3	CONTROL1	14	3,3	CELLS	64,8	8,9
ANDRO2	13,5	2,7	DWARF2	12	7,9	CONTROL2	14,9	5,2		56	6,1
ANDRO3	11,3	1,6	DWARF3	13	2,9	CONTROL3	23,2	4,4		56,4	5,8
ANDRO4	8,9	2,7	DWARF4	12,6	3,1	CONTROL6	11,9	1,6		59,07	6,93
ANDRO5	13,9	1,5	DWARF5	11,7	1,7	CONTROL5	21,7	6,1			
ANDRO6	13,7	2,5	DWARF6	10	1,6	CONTROL7	13,2	2,8			
ANDRO7	14,3	3,1	DWARF7	9,4	1,4	CONTROL8	11,4	3,9			
ANDRO1	14,4	5,7	DWARF1	12	2,6	CONTROL1	12,3	1,8			
ANDRO2	12,8	3,3	DWARF2	13,6	3	CONTROL2	12,2	2,3			
ANDRO3	11,2	2,3	DWARF3	18,7	4,5	CONTROL3	18,5	4,1			
ANDRO4	12,3	3,2	DWARF4	25,2	8,9	CONTROL6	12,9	2,4			
ANDRO5	10	2,4	DWARF5	14,1	6,5	CONTROL5	18,8	4,9			
ANDRO6	12,9	2	DWARF6	13,1	2,6	CONTROL7	17,3	3,7			
ANDRO7	14,4	3,4	DWARF7	10,6	1,4	CONTROL8	11,7	2,6			

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Telomerase Activity in Androgenetic Rainbow Trout with Growth Deficiency and in Normally Developed Individuals

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Abstract

Role of telomerase in specimens with retarded growth (dwarfs) has not been thoroughly examined to date. Considering that some of the fish species show correlation between somatic growth and activity of telomerase, it has been tempting to assume that pattern of telomerase activity in specimens with retarded growth and these with normal growth rate may vary. In the present research, telomerase activity has been examined in liver, skin, and muscles in the androgenetic rainbow trout (*Oncorhynchus mykiss*) with growth deficiency and their normally developed siblings. Among the examined organs, the liver showed the highest telomerase activity in all studied fish, what may be linked to the enormous regeneration capacity of the liver tissue. Although dwarf specimens examined here displayed significantly lower body size and weight they did not exhibit any significant differences in the telomerase activity measured in liver and muscle when compared to the rainbow trout without growth deficiency. In turn, telomerase activity in skin was significantly upregulated in the normally developed androgenotes. The present study indicates that dwarfism in the androgenetic rainbow trout is neither associated with ceased telomerase activity nor its decrease throughout the ontogenetic development.

Keywords: telomerase activity, growth, androgenotes, dwarf, rainbow trout

Introduction

Telomeres are eukaryotic terminal chromosomal regions composed of tandemly repeated DNA sequences associated with specific proteins. In vertebrates, telomeric DNA sequence is TTAGGG motif that extends in a 5′ to 3′ direction from double-stranded DNA to a single-stranded overhanging region.[1] Telomeres protect chromosomes from degradations and fusions,[2,3] ensure proper chromosome topology in the nucleus, and are able to silence expression of genes located adjacent to the telomeric region.[4,5] Telomeres shorten after each round of the cell division and this loss may be compensated by telomerase, a ribonucleoprotein enzyme composed of telomerase reverse transcriptase subunit (TERT) that adds telomeric DNA sequences to the 3′ end of telomeres using an integral RNA component (TR, telomerase RNA) as a template.[1]

Telomerase has been detected in all eukaryotes including vertebrates, however, its activity varies sometimes considerably between species. Generally, in mammalian species including humans telomerase is mostly absent or extremely downregulated in the somatic tissues/cells and its expression is observed exclusively in the highly proliferating cells including germ line cells and stem cells.[6,7] Moreover, upregulated telomerase has been reported in more than 90% of cancers and even up to 99% of the tumor originated cell lines.[8–10]

In contrast to humans, telomerase in mice,[11] birds,[12] fish,[13–19] and in the aquatic invertebrates[14,20] is active in the somatic tissues. The pioneer research on rainbow trout (*Oncorhynchus mykiss*) evidenced significant levels of telomerase activity in the kidney, liver, skin, heart, muscle, and brain irrespective of the fish age and size.[13] Studies on several model fish species exhibit a high correlation between TERT

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gene expression and variation in the telomerase activity, suggesting that telomerase activity in fish might be primarily regulated at the transcriptional level and not at the protein modifications stage.[18,19]

Observed levels of telomerase activity in fishes are mainly age and size dependent, usually displaying temporal increase during early developmental stages that are characterized by the fast somatic growth and accelerated cell proliferation.[16,21] Upon maturity, telomerase activity varies between species and organs and can decrease, sustain, or increase.[14,15,19,21] Levels of the telomerase activity in particular tissues are largely species-specific and reflect differential proliferative demands of cells. For instance, in zebrafish, platyfish (*Xiphophorus maculatus*), and in *Nothobranchius furzeri* lower activity of telomerase is detected in organs with the differentiated cells that are at the post mitotic state, such as brain and higher activity is reported in the undifferentiated cells for example, in gonads.[21–23]

Taking into account that in some of the fish species correlation between somatic growth and activity of telomerase in the somatic tissues has been observed,[16,19] it is tempting to assume that pattern of telomerase activity in the specimens with growth deficiency (like dwarfs) and those with the normal growth rate may vary. Dwarfism is a condition characterized by body size reduction that occurs in humans[24] and in animals[25] including fishes.[26–28] Dwarfism may be caused by genetic disorders (achondroplasia), growth hormone deficiency, or malnutrition.[24,29] Individuals with the growth retardation may have reduced but proportionate body or reduced body size may be accompanied by the skeletal abnormalities. Individuals with growth deficiency and body deformations have been observed among fish from wild stocks and from aquaculture.[30] Remarkably increased ratio of dwarf specimens has been reported among homozygous doubled haploids (DHs) produced by induced androgenesis and mitotic gynogenesis in salmonid fish species.[31–33]

As growth retardation makes dwarf fish interesting objects to study the roles of genetic and physiological factors responsible for this issue, the main goal of the present research was to examine activity of telomerase in androgenetic rainbow trout with growth deficiency and normally developed individuals.

Materials and Methods

The study was carried out in strict accordance with the recommendations in the Polish ACT of January 21, 2005 of Animal Experiments (Dz. U. of. 2005 No 33, item 289). The protocol was approved by the Local Ethical Committee for the Experiments on Animals in Bydgoszcz.

Fish origin and sampling

Studied rainbow trout originated from the spring spawning heterozygous Rutki strain and from the homozygous DHs strain produced within induced androgenesis using gamete donors originated from the Rutki strain (Fig. 1).[34] Fish were reared under routine conditions in the Department of Salmonid Research (DSR), Inland Fisheries Institute in Olsztyn (IFI), Rutki, Poland.

In total, fifteen 1-year old rainbow trout including five androgenotes with growth deficiency (dwarfs) ($_{d}$DH), five normally developed androgenotes ($_{n}$DH), and five heterozygous individuals from the control group (C) were sampled on July 20, 2020. Fish were sacrificed by overdose of the clove

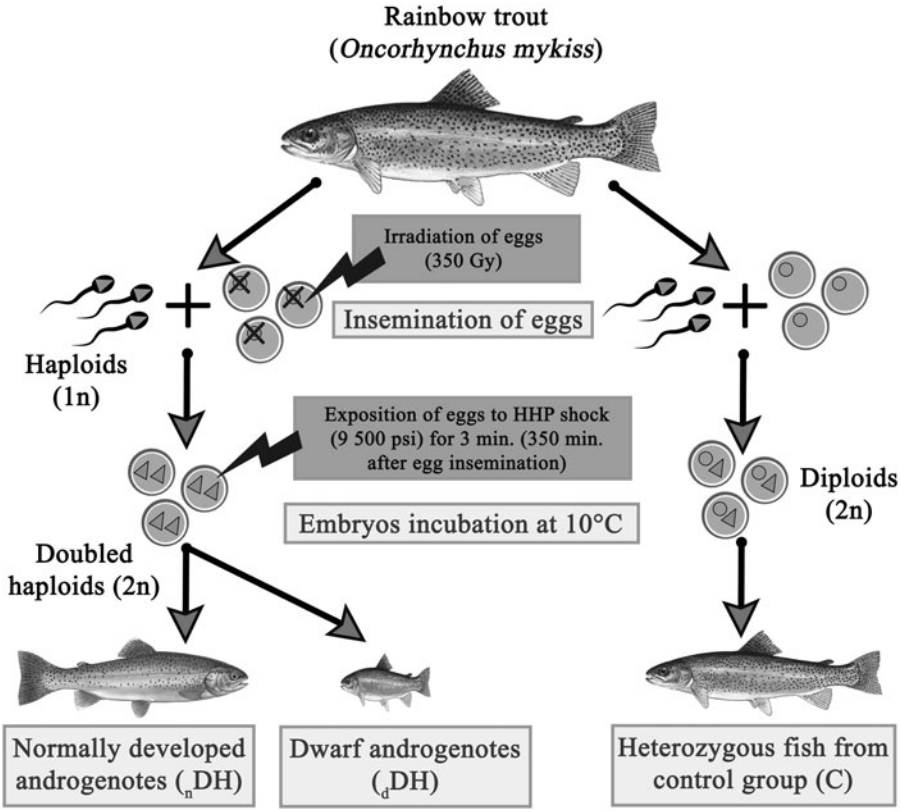

FIG. 1. The graphical summary of artificial androgenesis induced in the rainbow trout (*Oncorhynchus mykiss*). HHP, high hydrostatic pressure.

Table 1. Body Weight and Length of Normally Developed Androgenetic Rainbow Trout (nDH), Dwarf Androgenotes (dDH), and Individuals from Control Group (C)

Individual ID	*Body weight [g]*	*Body length [cm]*
$_dDH_2$	49.8	17.9
$_dDH_4$	46.9	17.1
$_dDH_5$	101.7	17.3
$_dDH_6$	76	19.5
$_dDH_7$	107.9	21.5
$_nDH_1$	164	24.7
$_nDH_2$	236.6	25.5
$_nDH_3$	215	23.2
$_nDH_4$	260	26.8
$_nDH_6$	202.1	25.6
C1	139.5	23.5
C2	226.5	28
C3	189.7	26.5
C4	179.6	26
C5	188.8	26.4

oil in a dose of 150 mg/L and their body weight and length were measured. Next, 100 g of liver, muscles, and skin were collected from each fish and immediately used for further analysis.

Analysis of telomerase activity

The telomerase activity assay was carried out in at least two replications for all collected tissues from the examined fish specimens. The analysis was performed using TeloTAGGG Telomerase PCR ELISA Kit (Roche Diagnostics GmBH, Mannheim, Germany) according to the manufacturer's instructions. The assay includes positive control, which is human, telomerase-positive embryonic kidney cell line 293. This technique is based on highly specific amplification of telomerase-mediated elongation products (TRAP) combined with nonradioactive detection following ELISA protocol.[35] The TRAP method is based on two-steps amplification, where at first telomerase adds telomeric repeats (TTAGGG) to the 3′ end of the biotin-labeled synthetic P1-TS-primer. Then the elongation products are amplified by PCR using the primers P1-TS and P2, generating PCR products with the telomerase-specific 6 nucleotide increments.

Under the ELISA assay, the obtained PCR products were denatured and hybridized to a digoxigenin-(DIG)-labeled, telomeric repeat-specific detection probe. Hybridized products were immobilized via biotin labeled primer to a streptavidin-coated microplate and detected with an antibody against digoxigenin (anti-DIG-POD) that was conjugated to peroxidase. An absorbance of colored reaction products was measured at 450 nm by a microplate reader (SpectraMax i3; Molecular Devices), using as a reference wavelength of 690 nm.

Statistical analysis

Data were analyzed using R software version 1.3.959. Normal distribution was tested by the Shapiro–Wilk test, whereas Levane's test was used to determine homogeneity of variances between analyzed groups of rainbow trout. Student *t*-test or Kruskal–Wallis test (according to the data distribution) was used to determine whether there were any significant differences ($p < 0.05$) in telomerase activity between organs in fish from the examined groups.

Results

The mean values of body weight and length of normally developed androgenotes ($_nDH$), androgenetic dwarfs ($_dDH$), and heterozygous individuals from the control group (C) equaled 215.54 ± 36.25 g and 25.16 ± 1.33 cm, 76.46 ± 28.33 g and 18.66 ± 1.85 cm, and 184.82 ± 31.06 g and 26.08 ± 1.63 cm, respectively (Table 1). Dwarf androgenetic rainbow trout displayed significantly lower body length and weight when compared to normally developed androgenotes and rainbow trout from the control group ($p < 0.05$). Particular dwarf androgenotes had visible morphological deformations such as humpback, kyphosis, and lordosis (Fig. 2). There were no significant differences in the body size between normally developed androgenetic trout and fish from the control group.

The liver was the organ with the highest level of telomerase activity that equaled 2.02 ± 0.55, 2.06 ± 0.36, and 2.52 ± 0.31 in normally developed androgenetic trout ($_nDH$), androgenotes with reduced growth ($_dDH$), and in the trout

FIG. 2. Body size of dwarf **(A)** and normally developed **(B)** androgenetic rainbow trout (*Oncorhynchus mykiss*).

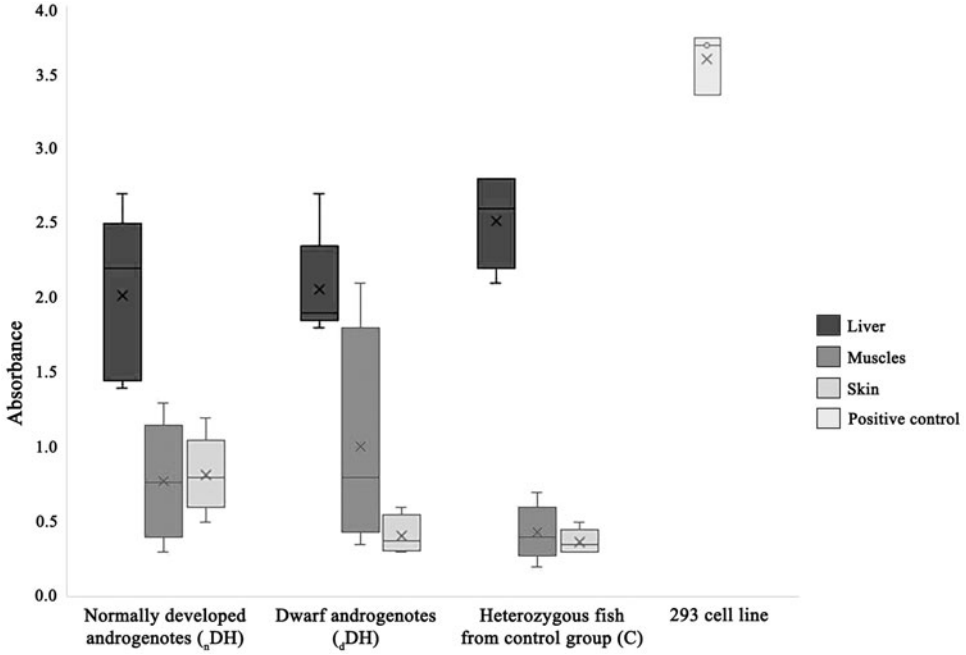

FIG. 3. Telomerase activity (expressed in absorbance) in the somatic tissues from the examined rainbow trout. 293 cell line: Human, telomerase-positive embryonic kidney cell line (3.61 ± 0.27).

from the control group, respectively (Fig. 3). The differences in the telomerase activity were insignificant ($p < 0.05$).

The activity of telomerase in the muscles of androgenetic trout from $_n$DH and $_d$DH groups was 0.78 ± 0.39 and 1.01 ± 0.76, respectively. The lowest activity of telomerase was observed in the muscles of fish from the control group (0.43 ± 0.19), however, the differences were not significant.

The activity of telomerase in the skin sampled from $_n$DH, $_d$DH, and heterozygous individuals from the control group was as follows: 0.82 ± 0.26, 0.41 ± 0.19, and 0.37 ± 0.08, respectively. The analysis showed significantly ($p < 0.05$) higher telomerase activity in skin from normally developed androgenetic trout when compared to dwarf specimens and heterozygous individuals from the control group. In the case of one dwarf rainbow trout, no telomerase activity was detected in the skin and in the muscles. The activity of telomerase in 293 cell line was sufficiently high (3.61 ± 0.27) which confirms good quality of performed analysis.

Discussion

In contrast to mammals, increased telomerase activity has been detected in the somatic tissues from varied fish species irrespective of their age.[13,16,36] Experiments conducted on several model and non-model fish species show that upregulated telomerase may be related to maintenance of the telomere length, somatic growth, and tissue regeneration.[15,17,21,37–40]

Here, 1 year old homozygous DH androgenetic rainbow trout with growth retardation and their normally developed homozygous and heterozygous siblings were examined to find out any differences in the telomerase activity among these fishes. Irrespective of the genetic status and fish appearance, the pattern of telomerase activity was similar with the highest abundance found in the liver tissue and the lower activity reported in the muscles and skin. Comparable activity of telomerase in dwarf and normally developed trout are paralleled with results of our previous experiment showing no differences in the telomere length between rainbow trout with growth retardation and those showing typical growth rate.[25] Equal length of telomeres observed in the rainbow trout siblings with disturbed and normal growth is thus explained by comparable activity of telomerase in those fish that compensate for any loss of the telomeric DNA.[26]

Although activity of telomerase has been confirmed in the muscles of all fishes studied to date under this regard,[13,15,37–39,41] its role in the fish somatic growth is not unambiguous. Some fishes are thought to exhibit indeterminate growth with the fastest growth rate observed during the early stage of life.[42] In adults, the growth rate slows down but never stops.[43] Thus, it was hypothesized first that indeterminate growth in fish might be associated with ubiquitous telomerase activity.[13–16,19] In marine medaka (*Oryzias melastigma*), early growth characterized by the rapid increase of the body mass is accompanied by upregulation of the telomerase activity in the muscles.[19] However, activity of telomerase changed upon maturity in these fishes. In sexually matured marine medaka, activity of telomerase was maintained in females but declined in males and was not related to the body mass.[19]

In the European hake (*Merluccius merluccius*) upregulation of TERT expression observed in the muscles and skin has been paralleled with increasing of the body size.[16] In turn, reduction of the telomerase activity in muscles in adults has been reported in Atlantic cod (*Gadus morhua*)[16] and rainbow trout[13] but not in zebrafish (*Danio rerio*).[41] These results suggest that telomerase may play some role during early development and growth in fish; however, different patterns of telomerase activity observed in adults between species indicate indefinite function of telomerase upon maturity.[19] Insignificant differences in the telomerase activity in muscles from rainbow trout with growth deficiency and their normally developed siblings confirmed that telomerase in dwarf trout is

not downregulated. On the other hand, significantly reduced abundance of telomerase observed in the skin of dwarf androgenotes may indicate that telomerase is somehow committed to processes related to growth in rainbow trout at least in the skin tissue, but further studies need to be performed to provide more information concerning this phenomenon.

In contrast to mammals, fish are considered as highly regenerative animals with capacity to regrow damaged heart, spinal cord, brain, and liver, among others.[14,21,40,44] A direct correlation between regeneration efficiency and telomerase expression observed in various fish species suggests that such upregulation of telomerase is not linked to their longevity or indeterminate growth but to the regeneration ability of injured, damaged, or amputated tissues.[14,17,36,41] Thus, the retained telomerase activity prevents telomere attrition during increased cell proliferating rates that accompany organ regeneration process. Liver is the organ that is famous for its capability to fully restore after acute injury for the mass and function. In several fish species, liver that was also confirmed to have a high regeneration capacity[45] is listed as the organ with relatively high activity of telomerase.[13,39,46] Similar has been observed in the present research where activity of telomerase was the highest in liver tissue in all groups of the sampled rainbow trout.

Conclusion

Our study indicated that telomerase in rainbow trout with growth deficiency is not downregulated at least in the muscles and in the liver. In contrast, skin exhibited decreased activity of telomerase in the dwarf rainbow trout. Unequivocal results concerning activity of telomerase in the dwarf rainbow does not verify the role of this enzyme in processes related to somatic growth in fish. In turn, increased abundance of telomerase observed in the rainbow trout liver may be related to its high regeneration capacity.

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Authors' Contribution

K.O. and L.P. designed and coordinated the study. L.P., A.B., and M.K. carried out the laboratory works and data analysis. M.K. performed the graphics preparations. All authors were involved in writing and editing of the article.

Disclosure Statement

The authors declare no conflicts of interest.

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Article

Telomerase activity in somatic tissues and ovaries of diploid and triploid rainbow trout (*Oncorhynchus mykiss*) females

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Abstract: Telomerase activity has been found in the somatic tissues of rainbow trout. The enzyme is essential for maintaining telomere length but also assures homeostasis of the fish organs, playing an important role during tissue regeneration. The unique morphological and physiological characteristics of triploid rainbow trout when compared to diploid specimens, make them a promising model for studies concerning telomerase activity. Thus, in this study, we examined the expression of the *Tert* gene in various organs of sub-adult and adult diploid and triploid rainbow trout females. Upregulated *Tert* mRNA transcription was observed in all the examined somatic tissues sampled from the triploid fish, when compared to diploid individuals. Contrastingly, *Tert* expression in the ovaries was significantly decreased in the triploid specimens. Within the diploids the highest expression of *Tert* were observed in the liver and in the ovaries from the sub-adult individuals. In the triploids, *Tert* expression was increased in the somatic tissues, while the ovaries exhibited lower activity of telomerase compared to other organs and was decreased compared to the ovaries from the diploids. The ovaries of triploid individuals were underdeveloped, consisting of only a few oocytes. The lack of germ cells, which usually are characterised by a high *Tert* expression, might be responsible for the decrease in the telomerase activity in the triploid ovaries. The increase of *Tert* expression in triploid somatic tissues suggests that they require higher telomerase activity to cope with environmental stress and to maintain internal homeostasis.

Keywords: telomerase; triploid; rainbow trout

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1. Introduction

Telomeres are non-coding regions of the genome that consist of tandemly repeated DNA sequences located at the ends of chromosomes. Telomeres not only protect coding fragments from loss during DNA replication, but also safeguard chromosomes against degradation and fusion, maintain their proper topology within the nucleus, and contribute to the transcriptional silencing of genes located in the vicinity of telomeric regions [1]. Due to the "end replication problem", telomeres shorten during each cell division, serving as a molecular clock that regulates the processes of cellular ageing and apoptosis. The loss of the telomeric tandem arrays may be compensated by telomerase, a ribonucleoprotein enzyme that consists of a catalytic subunit with reverse transcriptase activity (TERT) and an RNA template (TERC) [2]. Though, telomerase expression in the somatic tissues of certain endotherms is largely absent or repressed, with the enzyme activity only being present in germ line cells, tumour cells and stem cells [3-5]. Thus, in mammals and birds, telomeres shorten in almost all somatic cells as the organism ages [1].

In fish, it has been demonstrated that telomere shortening does not necessarily occur during ontogenesis and its dynamics may vary depending on the species [6-9]. This phenomenon is attributed to the telomerase activity that in the ectothermic organisms including fish is observed in the somatic cells irrespective of the organisms' age and size [6, 7, 9, 10, 11-14] and may prevent excessive telomere shortening during the period of rapid growth [6, 9].

Moreover, upregulated *Tert* expression is observed in fishes' regenerating tissues, indicating its crucial role in the healing process of body injuries [6, 11, 15-17]. The knock-out of the *Tert* gene using CRISPR/Cas9 in killifish (*Nothobranchius furzeri*) resulted in reduced fertility, atrophic testes, and ovaries, highlighting the significance of telomerase activity in maintaining organ and tissue homeostasis [18]. Research on several model fish species have demonstrated a high correlation between expression pattern of *Tert* gene and variation of telomerase activity what suggests that telomerase activity in fish is primarily regulated at the transcriptional level and not at the level of protein modifications [13, 14].

Rainbow trout (*Oncorhynchus mykiss*) is one of the most extensively farmed salmonid species worldwide. In 2020, its total production amounted to approximately 960,000 tonnes, making it the second most important species in aquaculture after Atlantic salmon (*Salmo salar*) [19]. The species is also a popular model animal in various scientific fields, including physiology, nutrition, toxicology, disease, ecology, genetics, and other fields [20-21]. Rainbow trout was the first fish species in which telomerase activity was confirmed in several organs/tissues regardless of the fish's age and size [10].

Spontaneous triploidization has been reported in rainbow trout, resulting from post-ovulatory oocyte ageing and the incidence of sub-lethally high temperatures during fertilisation and early zygote development [22-26]. Additionally, artificial triploidization can be induced in this species by dispermic fertilisation of a haploid egg [27], mating tetraploid and diploid individuals [28-30], or by exposing eggs to chemical (colchicine, ether, deuterium oxide, and specific enzyme inhibitors) or physical (sub-lethal temperature or high hydrostatic pressure) shocks that applied shortly after fertilisation prevent extrusion of the second polar body and result in the development of autotriploid embryos [31, 32]. The nuclear genome of the autotriploid embryos is composed of haploid sets of chromosomes originating from egg, sperm and the second polar body [33]. The additional set of chromosomes in triploids causes cytogenetic incompatibility, impairing proper gonadal development and gamete production [34]. As a result, triploid rainbow trout females (XXX) have underdeveloped ovaries with a low number of usually aneuploid oocytes, rendering them unable to produce eggs [35]. The reproductive sterility of triploid rainbow trout females makes them an attractive subject for aquaculture production, as they are not affected by the decline in growth rate and decreased meat quality commonly observed in diploids after sexual maturation [36, 37]. Moreover, sterile triploids cannot interbreed with wild fish populations if they should escape from fish farms or are introduced into open waters for recreational purposes [35, 38]. Triploidization has been also used as an effective sterilisation method for transgenic and potentially invasive fish species [39-42]. Triploid rainbow trout embryos have been used in reproduction studies as recipients for the interspecific transplantation of primordial germ cells to produce Atlantic salmon gametes [43]. On the other hand, it is commonly observed that triploid fish are more sensitive to sub-lethal external conditions, such as high temperatures and hypoxia, exhibiting reduced survivability and an increased incidence of skeletal malformations in comparison to their diploid counterparts [44].

Multiple studies have demonstrated that triploid rainbow trout can be an excellent model for investigating the mechanisms of gene expression regulation in autopolyploid organisms [45-48]. The unique morphological and physiological characteristics of triploid rainbow trout, including increased cell size, number of alleles/heterozygosity, sterility, continuous growth, and susceptibility to external conditions when compared to diploid specimens, make them a promising model for studies concerning telomere length dy-

namics and telomerase activity. Recent examinations have exhibited age-related changes in the telomeric DNA length in diploid and triploid rainbow trout females [8]. However, to the best of our knowledge, no information regarding the expression of the *Tert* gene in the somatic and reproductive tissues of polyploid vertebrates has been published to date. Therefore, the aim of this research was to explore the dynamics of telomerase expression in diploid and triploid rainbow trout females across somatic and reproductive organs.

2. Materials and Methods

2.1. Rainbow trout stocks, origin and maintenance

All-female diploid and all-female triploid stocks of rainbow trout were produced using gametes originating from breeders from a spring spawning Rutki strain kept in the Department of Salmonid Research (DSR) of the Inland Fisheries Institute (IFI) in Olsztyn, Rutki, Poland. For this purpose, eggs of rainbow trout were fertilised with milt from neo-males (XX), sex-reversed genetic females. To produce triploid stock of rainbow trout, a standard protocol that involves the application of a 3-minute-high hydrostatic pressure (HHP) shock (9000 psi) 35 minutes after egg insemination using a TRC-APV electric/hydraulic device (TRC Hydraulics Inc. in Dieppe, Canada) [34] was used. Both diploid and triploid rainbow trout individuals were reared separately under the same husbandry in the hatchery plastic tanks (1m^3) (first year) and outdoor in the rectangular (10m3) (second year) and circular concrete ponds (56m^3) (third year of rearing). The fish were fed daily and feeding rates were adjusted to their growth and diurnal temperature. Detailed information on environmental conditions during fish rearing have been described by Panasiak et al. (2020) [8]. Diploid and triploid females within the second (sub-adults) and third (adults) year of life were randomly chosen and sacrificed by an overdose of MS-222. For examination of telomerase expression and the histopathology of ovaries, five specimens from each age and ploidy stock were sampled. Cytogenetic analysis was applied for the ploidy confirmation.

2.2. RNA extraction and analysis of rainbow trout telomerase (Tert) expression

Liver, spleen, muscles, gills, and ovaries were sampled and immediately submerged in RNAlater (Thermo Fisher Scientific, USA). Preserved samples were kept at -20°C until further analysis. Total RNA was extracted using the Bead-Beat Total RNA Mini kit (A&A Biotechnology, Gdańsk, Poland), following the manufacturer's instructions. Residual DNA in the extracted RNA samples was removed using the Clean-Up Concentrator kit (A&A Biotechnology, Gdańsk, Poland). RNA concentration and purity were measured using a NanoDrop One spectrophotometer (Thermo Scientific, USA), and RNA integrity was assessed by 1% agarose gel electrophoresis. The obtained RNA samples were immediately processed further.

Purified total RNA samples of satisfactory quality were used to synthesise cDNA using the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, USA). The reaction mixtures were prepared in a total volume of 20 µl, composed by 1X Reaction Buffer, 5 µM of Random Hexamer primer, 1 µM of dNTP Mix, 20U of RiboLock RNase Inhibitor, 20U of RevertAid M-MuLV RT reverse transcriptase and 1 µg of RNA sample. The reverse transcription reactions were carried out in a Mastercycler® X50a (Epppendorf, Germany). The samples were incubated for 5 minutes at 25°C, followed by 60 minutes at 42°C, and terminated by heating at 70°C for 5 minutes. The obtained cDNA samples were diluted 1:10 in DEPC-treated water and stored at -20°C for further analysis.

The primer sequences for the *Tert* gene were designed based on genetic information deposited in the GenBank (accession numbers: KC204724-32, XR_005041402, HM852030, release 101) and Ensembl (accession number: ENSOMYG00000034782, release 109) databases. All available isoforms and splice variants of the *Tert* gene were taken into consideration before primer construction. The primers were designed with the Primer Blast

designing tool (NCBI) with default parameters, including the primer location on the exon-exon junction and a qPCR product length of approximately 100 bp.

Real-time PCR analysis was performed using designed primers for the *Tert* gene (Forward: 5′-CTCTTCCATCACCCCTGCTC-3′, Reverse: 5′-CCCCACTCACATCCACCTTG-3′). β-actin (*Actb*) was chosen as the housekeeping gene due to its proven stable expression level across different tissues (Forward: 5′-GCCGGCCGCGACCTCACAGACTAC-3′, Reverse: 5′-CGGCCGTGGTGGTGAAGCTGTAGC-3′) [49]. The qPCR was carried out separately for housekeeping and target genes by a Cielo 6 Real-Time PCR System (Azure Biosystems, Italy) using the PowerTrack SYBR Green Master Mix (Applied Biosystems, California, USA). Reaction efficiencies were estimated from the slopes of the standard curves made of 5-point, and 10-fold serial cDNA dilutions starting from 10 ng/ul. The optimal reaction conditions for both assays displayed an efficiency between 99-101%. The qPCR reaction mixtures were prepared in a total volume of 10 µl, consisting of 1X PowerTrack SYBR Green Master Mix, 0.5 µM (*Tert*) and 0.15 µM (*Actb*) of each primer and 1µl of cDNA. The Real-Time PCRs were run in triplicates with the following thermal cycling conditions: an initial polymerase activation step at 95 °C for 5 min, followed by 35 cycles of 95 °C for 30 s (denaturation), 20 s at 62 °C (primer annealing), and 72°C for 15s (elongation). During each run, negative controls using pure water and non-transcribed RNA were used to exclude contamination. Analysis of the melting curve (60–95 °C) at the end of each run concluded the protocol. Fluorescence data were collected after the elongation step and in 0.1 °C steps on the melting curve. No splice variants were observed in the analysed organs. The relative expression was calculated based on the difference between Ct values for reference and target genes, using the Livak and Schmittgen equation [50].

2.3. Histological preparation

Ovaries were removed and fixed with Bouin's solution. After preservation in Bouin's solution, fragments of the ovary tissue samples were dehydrated in 70% ethyl alcohol, treated with xylene, and submerged in paraffin blocks. Slices 4–5 µm thick were cut using a LEICA RM 2165 rotational microtome (LEICA Microsystems, Wetzlar, Germany) and stained with the HE (haematoxylin-eosin) topographic method [51]. Histological analyses of cross-sections to determine the shape, size and type of germ cells present in the gonads were performed using a LEICA DM 3000 transmission light microscope and the micro image computer analysis software LEICA QWin Pro (LEICA Microsystems AG, Heerbrugg, Switzerland). The nomenclature of cellular structures and germ cells in the analysed gonads was adapted according to Brown-Peterson et al. (2011) [52].

2.4. Ploidy confirmation: preparation of the metaphase spreads and microscopic analysis

Somatic metaphase chromosomes were prepared from the cells of a cephalic kidney, a primary fish lymphoid organ. Portions of the cephalic kidney were removed, placed in tubes with 5 mL of KCl (0.075 M) and 10 µl of 0.3% colchicine (Sigma–Aldrich, St. Louis, MO, USA) solution and incubated for 20 min. at room temperature. Fragments of tissues were then macerated with scissors, dissociated by pipetting to obtain homogenous cell suspensions and left for hypnotisation for another 45 min. Next, 10 drops of freshly prepared ice cold fixative (methanol: acetic acid, 3:1) were added to the tubes with cell suspensions. After 1 min, the tubes were filled with the fixative up to 8 mL and centrifuged at 160 × g for 10 min. Then, the supernatant was tossed out and the cell pellet was resuspended in freshly prepared fixative. Samples were kept at -20 °C for 30 min. The fixative was changed three times. After the final centrifugation, the supernatant was replaced by a freshly prepared fixative (1–2 mL). Microscope slides were prepared by placing one drop or two drops of the cell suspension from a height of about 30 cm onto a clean slide and left to dry.

The slides with the chromosomes were mounted using antifade solution Vectashield containing DAPI (4',6-diamidino-2-phenylindole) (1.5 μg/ml) (Vector, Burlingame, CA, USA) and analysed using the BX53 Olympus microscope (Olympus, Japan) equipped with epifluorescence, an appropriate filter set and a dedicated 5 M CMOS camera (Applied Spectral Imaging, Israel). Images of the metaphase chromosomes were captured and the electronic processing of the images was performed using GenASIs 8.1.1 software (Applied Spectral Imaging, Israel).

2.5. Statistical analysis

The fold change expression values of *Tert* relative to the reference gene were analysed using R software version 2022.12.0 (20.02.2023). Normal distribution was assessed using the Shapiro-Wilk test, and the homogeneity of variances between the analysed groups of rainbow trout was determined using Levene's test. The student t-test or Kruskal-Wallis' test was used, based on the data distribution, to determine significant differences ($P<0.05$) in the telomerase expression between the organs of the fish from the examined groups.

3. Results

3.1. Confirmation of ploidy – cytogenetic examination

The chromosome number in rainbow trout from the diploid stock varied from 59 to 63 while fish from the triploid stock exhibited 89 to 94 chromosomes. The variation in the chromosome numbers observed here originated from Robertsonian translocation, which in rainbow trout is a common characteristic [53, 54]. The differences observed in the number of chromosomes showed that the studied fish differed in terms of their ploidy.

3.2. Histology of gonads

Macroscopically, the gonads of the diploids were composed of paired, correctly shaped, similar-sized lobes located cephalically in the peritoneal cavity, embedded just below the wall of the swim bladder (Fig. 1A). In contrast, in the triploid fish these structures were characterized by extremely thin, poorly demarcated bands of tissue adhering parallel along the ventral port of the swim bladder wall (Fig. 1B).

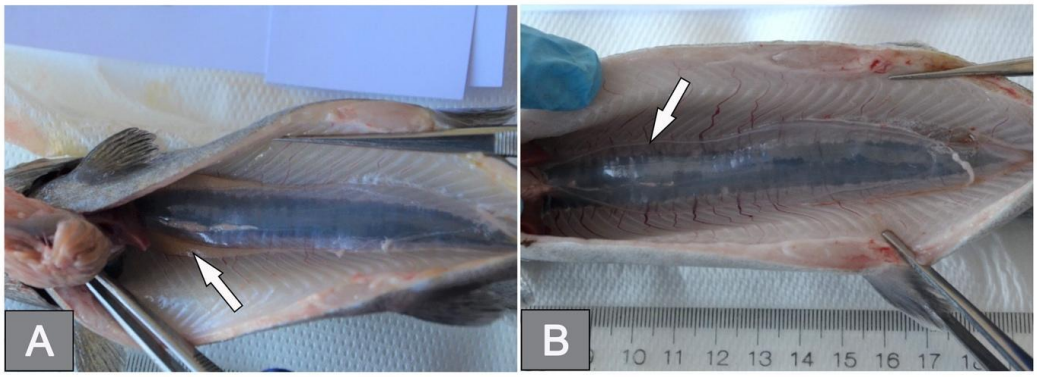

Figure 1. Macroscopic structure of the rainbow trout gonads: (A) diploid female; (B) triploid female.

Histological analysis of the gonads from the diploids revealed the presence of only ovaries filled with oocytes at the previtellogenesis stage, accompanied by single oogonia nests. Some of the fish were characterized by the presence of distinct ovarian lamellae, typical of salmonid ovaries, within the structure of the gonads (Figure 2A), while others lacked any elements separating groups of previtellogenic oocytes (Figure 2B). In the sub-adult fish, the presence of a small number of vitellogenic oocytes at the initial stage of

vacuolization was noted (Figure 2C). In the ovaries of the triploid individuals, two types of ovarian tissue structures were observed on the histological sections. In some examined specimens the interior of the organ structure consisted exclusively of the somatic connective tissue cells (fibrocytes) and surrounding fibrous elements enclosed in a connective tissue structure surrounded by several layers of differentiated epithelial cells. In the cross sections of such ovaries, faintly outlined structures resembling ovarian lamellae were observed (Figure 2D). In other triploids, the structure of the gonads was disrupted and the ovaries were filled with connective tissue elements with single (Figure 2E) or few previtellogenic oocytes (Figure 2F) located in the central part of such gonads.

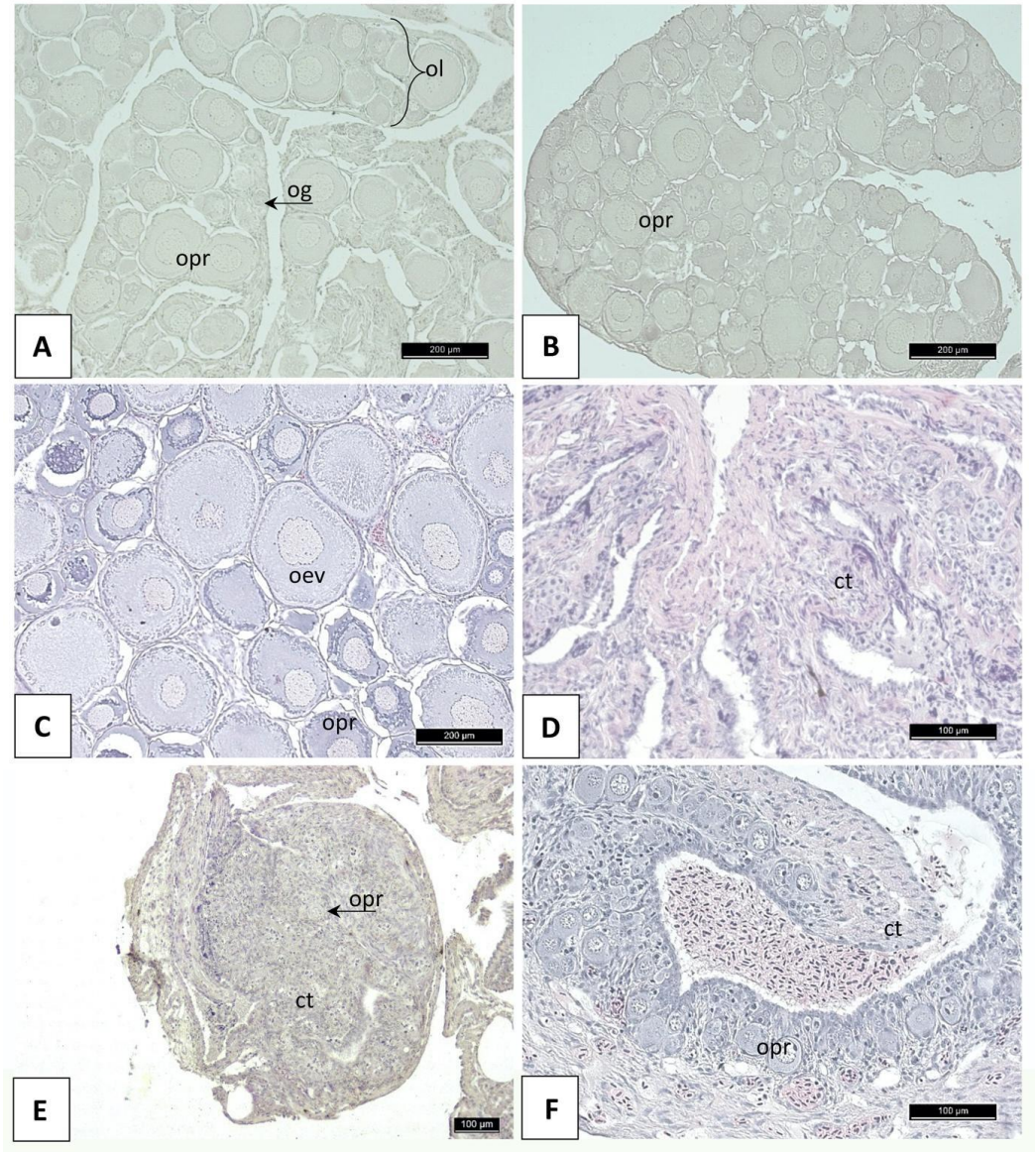

Figure 2. Histological cross-sections of the rainbow trout gonads: (A) the ovary of a diploid with typical ovarian lamellae; (B) the ovary of a diploid individual formed of ovarian lamellae; (C) the ovary of a diploid fish; (D) the sterile ovary-like organ of an individual filled with connective tissue cells and fibrous elements; (E) the ovary-like gonad of a triploid individual with a single previtellogenic oocyte located in the central part of the organ; (F) the ovary-like gonad of a triploid individual with a few previtellogenic oocytes. Description: ct – connective tissue elements, og – oogonia, oev – early vitellogenesis oocyte; ol – ovarian lamina, opr – previtellogenic oocyte.

3.3. Expression of the Tert gene

Real-time PCR was used to quantify the *Tert* mRNAs, which were detected in all the analysed organs. Generally, significantly (P<0.05) increased expression of the *Tert* gene was observed in all the examined somatic tissues from triploid specimens when compared to the diploids. A contrasting situation was reported in the ovaries sampled from triploid individuals, which showed a decreased expression (Fig. 2.). Upregulated *Tert* expression was detected in muscles sampled from adult (3-year-old) rainbow trout compared to sub-adult (2-year-old) fish; however, the observed differences were significant (P<0.05) only among diploids. In the gills, no significant differences in *Tert* expression were reported between sub-adult and adult fish. In the liver, significantly (P<0.05) higher *Tert* expression in triploids compared to diploids was detected only in the adult trout, while transcription levels of *Tert* gene in the sub-adults were significantly lower in the triploids than in the diploids (Fig. 2). The *Tert* expression level in the spleen was essentially increased in adult triploids, when compared to all other fish groups. No differences in *Tert* expression levels were observed between sub-adult diploids, triploids and adult diploid trout. In the ovaries, the transcription level of *Tert* mRNAs in diploids was significantly higher (P<0.05) than in the triploids, regardless of the age of the fish. Additionally, sub-adult individuals exhibited a slightly higher expression of *Tert* than adult specimens in both the diploid and the triploid groups, though the differences were not significant (P<0.05).

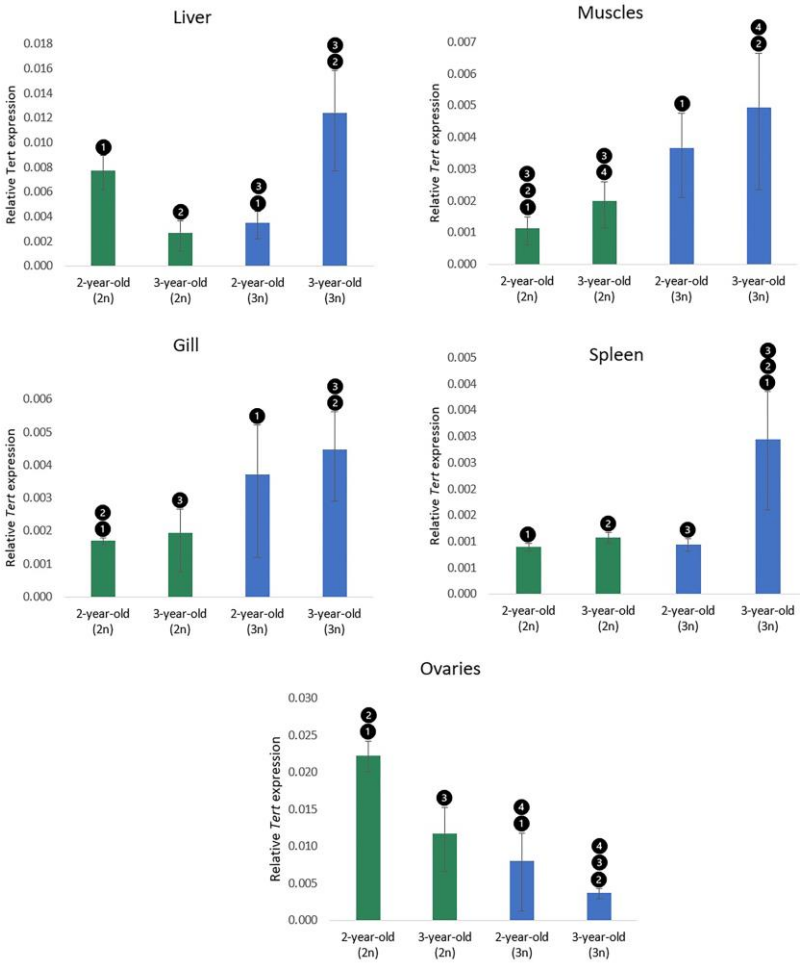

Figure 3. Relative *Tert* expression in the organs of rainbow trout. The same numbers indicate statistically significant (P<0.05) differences between groups. Values are presented as fold changes relative to the reference gene (*β-actin*). The measure of variation is derived from respective SEM of the Ct values.

4. Discussion

Compared to their diploid counterparts, triploids exhibit three fundamental differences: they are more heterozygous, they have fewer larger cells in the tissues and their gonadal development is disrupted [34, 55]. Despite the commonly held belief that larger cells have lower metabolic rates per unit of mass [56], the reported energy metabolic and oxygen consumption rates in triploid fish are usually similar or even higher than those in diploid individuals [34, 55]. Likewise, the developmental rates of triploids are generally comparable to those of diploids, although in some cases they may be slightly lower or higher [34, 55]. However, after the sexual maturation of diploid fish, triploids tend to exhibit faster growth as they do not invest energy for gonadal development [33]. Triploids also display a higher ratio of malformation incidences compared to diploids that is based on their genetic perturbations resulting from the ploidy change, the detrimental effects of physical shock applied for triploidization and distinct nutritional requirements [57, 58]. A depleted resource transport capacity (especially of oxygen) in larger cells, attributed to longer diffusion distances and reduced membrane surfaces, limits triploids' aerobic energy budget and abilities to accumulate energy reserves [59]. Triploids may also display an increased mortality rate as they are less resistant to chronic hypoxia and high temperatures than diploids. Nevertheless, under optimal conditions, both triploids and diploids actually tend to display similar mortality rates [34, 55].

Despite large cellular architecture and physiological differences between triploids and diploids, the effect of triploidy on the gene expression is surprisingly smaller than expected as triploid and diploid fish have usually comparable levels of expression in their somatic tissues (a dosage compensation phenomenon) [60-65]. Only a small number of genes may be subjected to both positive and negative dosage compensation in triploid fishes [66]. Most of the up or down regulated genes reported so far in the triploids are those regulating metabolism and stress responses, which is hypothesised to be associated with the higher susceptibility of triploids to hypoxia, and thermal and oxidative stresses when compared to diploids [34, 55, 63, 64].

Research on *Tert*-/- knock-out zebrafish mutants revealed severe histopathological abnormalities in their testes, liver, intestine, gills, pancreas, kidney and muscle tissues which suggests that activity of telomerase in fish irrespective of their age plays an important role in the maintenance of the tissue homeostasis [67]. Thus, cellular and physiological differences between diploid and triploid fish including a larger size of cells, potentially changed standard metabolic rate and decreased resistance for demanding conditions, may thus require increased telomerase activity in triploids to sustain proper physiology of their tissues. Rearing conditions optimal for the diploid rainbow trout examined here, might have been more demanding for the triploid individuals, so the maintenance of the tissue homeostasis required increased telomerase expression.

Telomerase expression and activity have been extensively studied in the muscles of various fish species [9, 10, 17, 68, 69]. However, the precise function of telomerase during somatic growth and connection with indeterminate growth of fish remains unclear. For instance, a study on the European hake (*Merluccius merluccius*) revealed a positive correlation between the levels of *Tert* transcription and body size, while opposite findings were reported in the Atlantic cod (*Gadus morhua*) and the rainbow trout [10, 70]. Frequently reported up-regulation of telomerase transcription and activity during early developmental stages in fish suggests its role in promoting tissue cellular proliferation [9, 10, 11, 14, 70]. Nevertheless, in our previous study, differences in telomerase activity in muscles from trout siblings showing normal or retarded growth were insignificant [71]. Differences in the expression of *Tert* in the muscles of sub-adult and adult diploid and triploid rainbow trout may reflect a disparity in energy expenditure between fertile and sterile individuals for the somatic growth. Triploid salmonid females do not sexually mature so they do not need to spend much energy on their gonadal development and gamete production as diploids do. However, elevated rates of hypertrophic growth in

triploids, which possibly demands greater energy expenditure, might be considered to explain the up-regulated expression of *Tert* in the muscles of triploid fish [55, 72].

The liver, spleen, and gills are organs in fishes that are known for their remarkable regenerative capabilities and resistance to oxidative stress [6, 11, 69]. The ability of these organs to regrow after injury or damage has been linked to increased telomerase activity [7, 11, 15-17]. Triploid rainbow trout have been found to be more resistant to carcinogenesis and have increased fin regenerative abilities when compared to diploid fish [73]. Our study's findings indicate that the increased regenerative abilities of triploids may be attributed among others to the upregulation of telomerase expression in their somatic tissues [35, 74]. Moreover, the increased telomerase activity observed in triploid rainbow trout might explain the higher resistance of the polyploid cells/tissues to the oxidative stress [75]. The liver serves as a vital organ for regulating organisms' energy metabolism, but it is also highly exposed to the toxic compounds and reactive oxygen species (ROS) that arise during metabolic processes [76]. In fish, the liver is the organ with the highest level of telomerase expression and activity, which is thought to be linked with the ability of this organ to cope with the oxidative stress [10, 71]. The significantly upregulated *Tert* expression in the liver of the sub-adult diploid fish when compared to the triploids might reflect a surge of the metabolic rate associated with the sexual maturation process and vitellogenin synthesis, among others. The decreased expression in *Tert* observed in the adult fish may be correlated with a lower liver metabolic rate as the process of gonadal maturation in triploids is accomplished. Nevertheless, further research is needed to explain the background of this phenomenon. Multiple studies demonstrated that triploids require higher gill irrigation rates to compensate for their lower respiratory efficiency and oxygen supply compared to diploids [54, 77]. The higher rate of opercular movement in triploids can potentially increase their exposure to environmental stressors, thus, up-regulated *Tert* transcription reported in the gills of triploid trout might play some role in maintaining its homeostasis and regenerative abilities. In turn, the significantly increased levels of telomerase expression in the spleen of adult triploid rainbow trout observed here may be linked with the increased synthesis and accumulation of erythrocytes observed in the triploid rainbow trout that was thought to be a compensatory response to lower oxygen supply [78].

Telomerase expression in mammalian adults is restricted to highly proliferating cells including female germline stem cells that can differentiate into oocytes. Moreover, telomerase activity has been confirmed in the oocytes at different stages of development [79,80] and in the ovarian granulosa cells that proliferate in the developing follicle and form a single layer around the oocytes [81-83]. In fish, expression of *Tert* varies significantly between tissues, and gonads are organs with the highest telomerase activity [6, 84, 85]. This is also observed in the diploid rainbow trout examined here. Telomerase plays an important role during fish ovarian development and egg production and maturation. *Tert* deficient fish are characterised by atrophied ovaries, reduced egg production and premature infertility [67, 18]. A dramatic decrease of telomerase activity has also been observed in the ovaries from triploid rainbow trout that are considered sterile. The ovaries in triploid rainbow trout are highly reduced with only a few primordial germ cells and several, usually euploid or aneuploidy, oocytes [34, 86-88]. The gonads of the triploid trout studied here were actually devoid of oocytes and consisted exclusively of the somatic connective tissue cells, mostly fibrocytes (Fig. 2) The sterility in triploid rainbow trout females results from the arrest of the oogonium development, failure of the meiosis, and lack of the interactions between oocytes and follicular cells. Moreover, in triploid ovaries, the development of follicular cells is inhibited and production of estradiol decreased, which in turn causes a decline in synthesis and secretion of the hepatic vitellogenin (VTG) [89], a major precursor of the fish egg yolk protein that is indispensable for the oocyte growth and maturation in fish. Moreover, oocytes in triploid rainbow trout may undergo apoptosis [90]. The presence of only a few germ cells and oocytes that are usually characterised by high telomerase activity may be therefore responsible for the

reduced expression of *Tert* observed in the ovaries from triploid rainbow trout females. Furthermore, it has been confirmed that telomerase is activated by oestrogen through the stimulating of *Tert* expression [91], hence a deficiency of estradiol observed in ovaries from triploid rainbow trout [92] may be also responsible for the decreased expression of *Tert*.

5. Conclusions

Ploidy compensation is known as an important process that ensures the equal distribution of protein production across different ploidy levels in organisms. Recent research on dosage compensation in polyploid fishes has shown that diploid and triploid individuals exhibit similar levels of gene expression in most analysed tissues, indicating compensation mechanisms are in place. However, some genes may show either positive or negative dosage compensation in triploid fish, with most up- or down-regulated genes in triploid salmonids being related to metabolism and stress response. The positive dosage compensation of the *Tert* gene expression in several organs of triploid rainbow trout females found in our study supports the notion that telomerase activity is essential in sustaining fish tissue homeostasis/biology. This study adds to our understanding of the relationship between the ploidy and telomerase expression in fish and suggests that telomerase may play a more significant role in the biology of triploid organisms than previously thought. The significant downregulation of *Tert* expression in the ovaries of triploid rainbow trout, as observed in our study, appears to be correlated with their atrophied state, decreased egg production, and infertility. The absence of oocytes in triploid ovaries, along with their inhibited follicular cell development might contribute to the recorded decline in telomerase expression. Nevertheless, further research is needed to explain the different patterns of telomerase expression between diploids and triploids across different organs reported here that will allow us to better understand the relationship between ploidy and *Tert* gene expression in vertebrates.

Supplementary Materials: Supplementary Table S1. Parameters of diploid and triploid rainbow trout body weight and length.

Author Contributions: Conceptualization, K.O. and L.P.; Methodology, K.O., L.P., P. H., M.K. and K. P.; Software, L.P.; Formal Analysis, L.P.; Resources, K.O. and S.D.; Writing – Original Draft Preparation, K.O., L.P., K.P., P.H and M. K.; Writing – Review & Editing, K.O., L.P., M.K.; Visualization, L.P., P.H.; Supervision, K.O.; Funding Acquisition, K.O. All authors have read and agreed to the published version of the manuscript.

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Informed Consent Statement: Not applicable.

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Conflicts of Interest: The authors declare no conflict of interest.

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Supplementary Table1 Parameters of diploid and triploid rainbow trout body weight and length.

Individual	Ploidy	Age	Body length [cm]	Body weight[g]
2.8	2n	2 year old	27	211
2.9	2n	2 year old	21.2	97
2.10	2n	2 year old	20.2	79
2.11	2n	2 year old	22.2	113
2.12	2n	2 year old	24.2	148
3.8	2n	3 year old	30	363
3.9	2n	3 year old	32.5	404
3.10	2n	3 year old	31.8	352
3.11	2n	3 year old	32	390
3.12	2n	3 year old	35.5	542
2.1	3n	2 year old	25.4	158
2.2	3n	2 year old	27.7	276
2.3	3n	2 year old	24.5	153
2.4	3n	2 year old	27.2	230
2.5	3n	2 year old	28.5	208
3.1	3n	3 year old	36	407
3.2	3n	3 year old	32	229
3.3	3n	3 year old	37.5	560
3.4	3n	3 year old	33	401
3.5	3n	3 year old	36.5	615

STATEMENTS OF CO-AUTHORSHIP

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AUTHOR CONTRIBUTION STATEMENT

I declare that in the manuscript:

Panasiak, L., Dobosz, S., Ocalewicz, K. (2020). Telomere dynamics in the diploid and triploid rainbow trout (*Oncorhynchus mykiss*) assessed by Q-FISH analysis. Genes, 11(7), 786.

My contribution involved conducting process of triploidization and providing care for the embryos, larvae, and adult fish from both triploid and diploid groups.

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My contribution involved preparation and culture of the L5178Y-R cell line and assisting in preparation of the manuscript for this publication.

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My contribution involved induction of androgenesis.

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My contribution consisted of conducting ELISA test, analyzing the data, and assisting in preparation of the manuscript for this publication.

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Sopot, 19.06.2023r.

AUTHOR CONTRIBUTION STATEMENT

I declare that in the manuscript:

Panasiak, L., Kuciński, M., Hliwa, P., Pomianowski, K., Ocalewicz, K. Telomerase activity in somatic tissues and ovaries of diploid and triploid rainbow trout (*Oncorhynchus mykiss*) females. Cells-2446865

My contribution involved optimizing the real-time PCR method and assisting in preparation of the manuscript for this publication.

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AUTHOR CONTRIBUTION STATEMENT

I declare that in the manuscript:

Panasiak, L., Kuciński, M., Hliwa, P., Pomianowski, K., Ocalewicz, K. Telomerase activity in somatic tissues and ovaries of diploid and triploid rainbow trout (*Oncorhynchus mykiss*) females. Cells-2446865

My contribution involved the histological preparation of the gonads and providing descriptions of the obtained results.

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AUTHOR CONTRIBUTION STATEMENT

I declare that in the manuscripts:

Panasiak, L., Kuciński, M., Błaszczyk, A., Ocalewicz, K. (2022). Telomerase activity in androgenetic rainbow trout with growth deficiency and in normally developed individuals. Zebrafish, 19(4), 131-136.

My contribution consisted of conducting the ELISA test, data analysis, and assisting in preparation of the manuscript for this publication.

Panasiak, L., Kuciński, M., Hliwa, P., Pomianowski, K., Ocalewicz, K. Telomerase activity in somatic tissues and ovaries of diploid and triploid rainbow trout (*Oncorhynchus mykiss*) females. Cells-2446865

My contribution involved designing primers, optimizing the real-time PCR method, conducting data analysis, and assisting in preparation of the manuscript for this publication.

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AUTHOR CONTRIBUTION STATEMENT

I declare that in the manuscripts:

Panasiak, L., Dobosz, S., Ocalewicz, K. (2020). Telomere dynamics in the diploid and triploid rainbow trout (*Oncorhynchus mykiss*) assessed by Q-FISH analysis. Genes, 11(7), 786.

My contribution included experimental design, induction of the triploid development, fish sampling, data analysis and preparation of the manuscript for this publication.

Panasiak, L., Szubert, K., Polonis, M., Ocalewicz, K. (2022). Telomere length variation does not correspond with the growth disturbances in the rainbow trout (*Oncorhynchus mykiss*). Journal of Applied Genetics, 63, 133-139.

My contribution included experimental design, fish sampling, data analysis, and manuscript preparation for this publication.

Panasiak, L., Kuciński, M., Błaszczyk, A., Ocalewicz, K. (2022). Telomerase activity in androgenetic rainbow trout with growth deficiency and in normally developed individuals. Zebrafish, 19(4), 131-136.

My contribution included experimental design, data analysis, and manuscript preparation for this publication.

Panasiak, L., Kuciński, M., Hliwa, P., Pomianowski, K., Ocalewicz, K. Telomerase activity in somatic tissues and ovaries of diploid and triploid rainbow trout (*Oncorhynchus mykiss*) females. Cells-2446865.

My contribution involved experimental design, fish sampling, preparation of the metaphase spreads, microscopic analysis, data analysis, and manuscript preparation for this publication.

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AUTHOR CONTRIBUTION STATEMENT

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My contribution involved collecting tissue samples from fish, conducting Q-FISH analysis, microscopic analysis, data analysis (including statistical analysis), and manuscript preparation for this publication.

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My contribution included collecting organs from fish, conducting ELISA tests, data analysis (including statistical analysis), and manuscript preparation for this publication.

Panasiak, L., Kuciński, M., Hliwa, P., Pomianowski, K., Ocalewicz, K. Telomerase activity in somatic tissues and ovaries of diploid and triploid rainbow trout (*Oncorhynchus mykiss*) females. Cells-2446865.

My contribution involved collecting organs from fish, RNA isolation, first-strand cDNA synthesis, conducting real-time PCR reactions, data analysis (including statistical analysis), and manuscript preparation for this publication.