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].

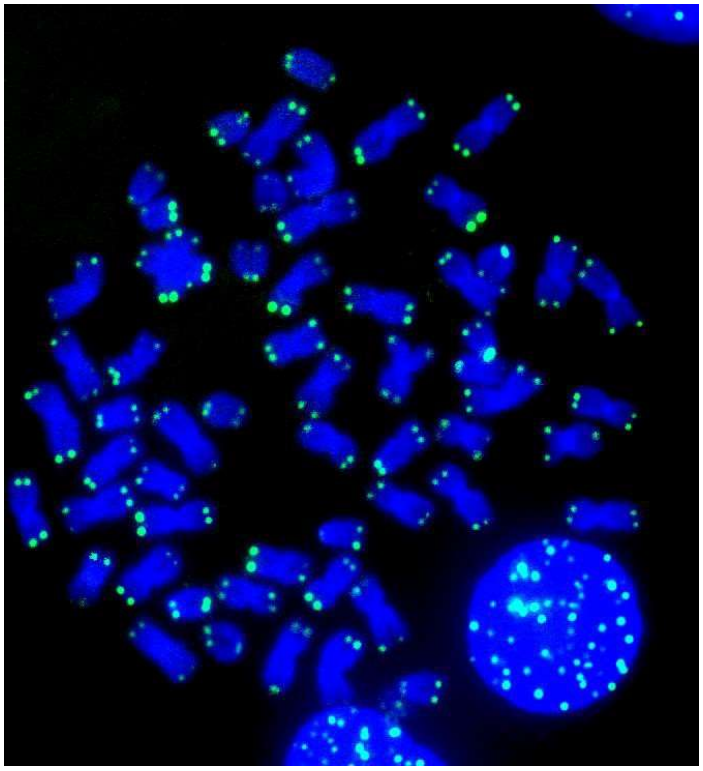

Figure 1 *Rainbow trout chromosomes hybridized with a PNA telomeric probe labeled with fluorescein isothiocyanate (FITC).*

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.

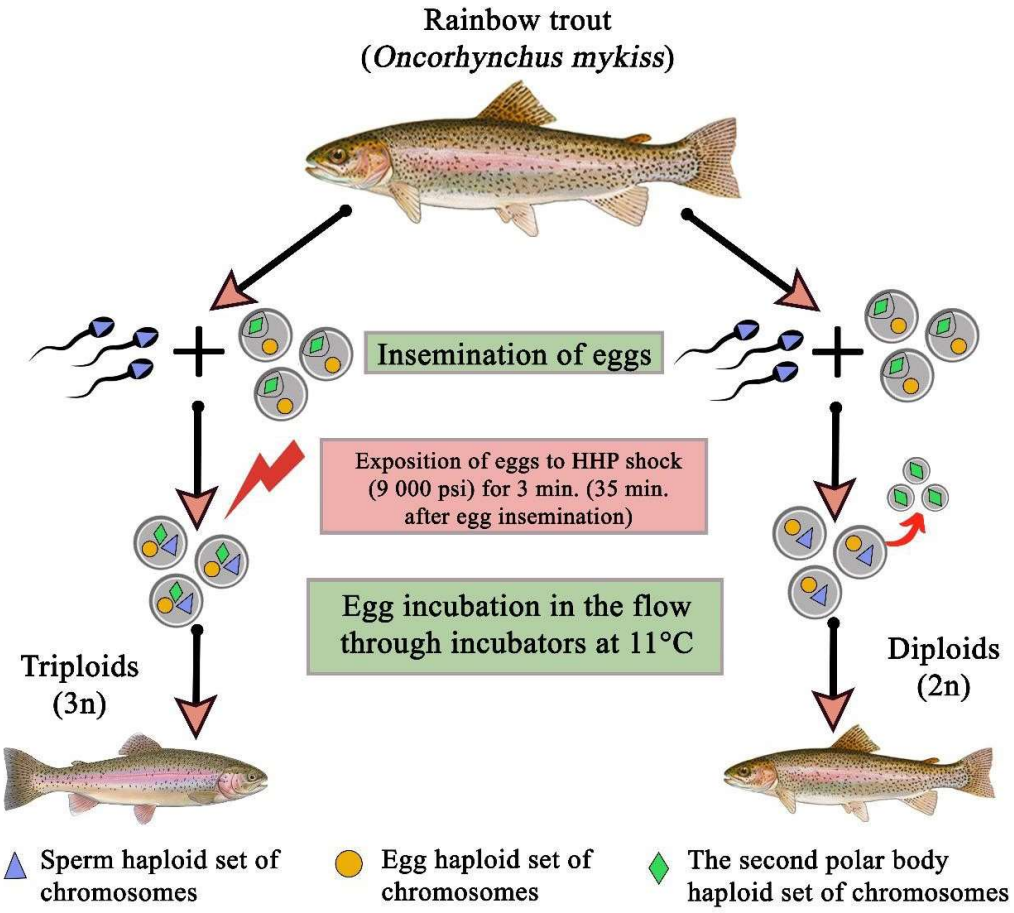

Figure 2 *The graphical summary of triploidisation induced in the rainbow trout*

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.

Summary

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.