Original Research Paper

Age-Dependent Variation of Telomere Length and DNA Damage in Chicken

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Abstract: Birds are unique models for molecular studies on aging processes and cellular senescence because of their slow rates of aging and comparatively long life spans among homoeothermic animals. The study aimed to examine the relative telomere length and amount of nuclear, mitochondrial DNA lesions in hens of different ages and to find out the correlation between these biomarkers of aging. The study was carried out on hens of crossbreed «High Sex Brown» of different age groups - 1, 75, 225, 330, 450 days old. The measurements of telomeres and DNA damage were done in red cells of hens using quantitative PCR technique. We found out that the amount of telomeric DNA reduced significantly with age. Telomere length ratio to control group (1 day old) made up: 0.88 ± 0.08 for 75 days old, 0.73 ± 0.07 for 225 days old, 0.66 ± 0.1 for 330-days old and 0.58 ± 0.12 for 450 days old. The amount of DNA lesions in both genomes had great, age-dependent increase. The relative quantity of lesions per 10 kb detected in the nuclear DNA were as follows: 0.17 ± 0.19 for 75 days old, 0.66 ± 0.21 for 225 days old, 1.02 ± 0.33 for 330-days old and 1.48 ± 0.45 for 450 days old and the relative quantity of lesions in mtDNA were as follows: 0.11 ± 0.06 for 75 days old, 0.84 ± 0.23 for 225 days old, 1.18 ± 0.3 for 330-days old and 1.49 ± 0.35 for 450 days old. The results obtained are of fundamental interest for aging processes, as well as could have an economic value for poultry farming.

Keywords: Aging, Chicken, Telomere Length, DNA Lesions

Introduction

The aging processes are being widely investigated in diverse taxa (Gomes *et al*., 2010; Baudisch, 2012) and among vertebrates aging of birds is of particular interest. For instance, birds, on average, live three times longer than mammals of equivalent body weight (Holmes *et al*., 2001). Birds have the paradoxical long life expectancy and slow rates of aging as compare with mammals, especially, taking into account such features of avian physiology, that adversely affect lifespan: elevated body temperature (about 3°C higher than in mammals), rapid metabolism processes (2-2.5 times higher), high blood glucose content (2-4 times higher) (Holmes *et al.*, 2001; Austad, 2011). Thus all these features make birds extremely interesting objects for studying the aging processes.

One of DNA structure parameters correlating with age is telomere length (Gomes *et al*., 2010). It has been found that telomere length of somatic cells decreases with age in various species of different taxa, for instance: Siberian sturgeon (Simide *et al*., 2016), turquoise killifish (Ocalewicz, 2013), nine-spine stickleback (Noreikiene *et al*., 2017), loggerhead sea turtle (Hatase *et al*., 2008), alligators (Scott *et al*., 2006; Xu *et al*., 2009) sand martins, dunlins (Pauliny *et al*., 2006), donkey, sheep, domestic cat (Gomes *et al*., 2010) and others. Many studies of avian telomere length variation with age were done on wild populations of birds (Pauliny *et al*., 2006; Criscuolo *et al*., 2009; Heidinger *et al*., 2012; Herborn *et al*., 2014). However, telomeres length has high interindividual variability and depends on many genetic and environmental factors (Mather *et al*., 2011;

Paul, 2011), hence for more accurate investigations of avian telomere length should be used model objects with similar genotype and environmental conditions. One of a useful model organism for study avian aging processes, including telomere length changing, is chicken (Swanberg *et al.*, 2010; Kim *et al.*, 2011). It is also worth noting, that investigation of chicken telomere-based mechanisms even may be more relevant to human biology than similar studies on commonly used rodent model organisms such as the laboratory mouse, which somatic cells have constitutive telomerase expression throughout the lifespan (Swanberg *et al.*, 2010). Another molecular hallmark of aging is DNA damage. There is no doubt that DNA lesions are accumulating with age (Takubo *et al.*, 2010). However, the rate of DNA lesions accumulation could vary widely in different cell types (Castro *et al.*, 2012; Sohn *et al.*, 2012) and differ between species (Meyer *et al.*, 2007; Hunter *et al.*, 2010). The amount of DNA damage also significantly depends on the type of genome: nuclear or mitochondrial (Wang *et al.*, 2010). Age-dependent variations of DNA lesions have been studied predominantly in mammals - rats (Hamilton *et al.*, 2001), mice (Wang *et al.*, 2009), rhesus monkeys (Castro *et al.*, 2012), human cells (Wang *et al.*, 2010), as well as invertebrate animal models - nematode (Meyer *et al.*, 2007) and fruit fly (Edman *et al.*, 2009). But there is a lack of similar research on birds. The study aimed to examine the relative telomere length and the amount of nuclear, mitochondrial DNA lesions in hens of different ages and to find out the correlation between these biomarkers of aging.

Materials and Methods

Husbandry and Sample Collection

The study was carried out on hens of crossbreed «High Sex Brown». «High Sex Brown» is highly performance crossbreed, with average hen-day egg production 80% and egg mass 50 g. The birds were maintained in the poultry cage systems - EURODENT-Parent (Big Dutchman (Germany)) with the microclimate conditions which are recommended by the manufacturer of High Sex Brown crossbreed: The temperature-20°C, the humidity-68% and light/dark cycles-9/15 h with the light intensity of 5 lux. The hens were fed with standard mixed fodder, which composition is presented in Table 1.

For research, we used blood samples from female chickens of different age: 1, 75, 225, 330 and 450 days old. 25 blood samples from each age group have were collected in heparinized tubes and centrifuged. Separated fractions of red cells were frozen in liquid N2 and then stored at -80°C until DNA extraction.

Table 1: The composition of feed ingredients

Item	Amount %
Wheat	15,95
Corn	45,00
Soybean meal, 46% CP	11,50
Sunflower meal, 36% CP	14,70
Sunflower oil	1,68
DL-Methionine (99%)	0,11
Fine limestone	9,10
Monocalcium phosphate	1,10
Sodium chloride	0,26
Sodium sulfate	0,10
Vitamin and mineral premix	0,50

DNA Extraction and Polymerase Chain Reaction Amplification

DNA extraction was performed from 40 uL of red blood cells fraction with spin column-based nucleic acid extraction kit - K-Sorb kit (Syntol, Russia). All the DNA isolation steps were done according to the manufacturer's instruction. The purity of the DNA was measured using NanoDrop 2000 (Thermo Fisher Scientific, USA). DNA quantity was measured using fluorometer QuantiFluor-ST (Promega, USA) and QuantiFluor dsDNA System (Promega, USA).

We used the method of quantitative PCR for measuring relative telomere length as described Heidinger *et al.* (2012) with modifications. Briefly, the relative telomere length of each sample was measured by determining comparative quantitation (ΔCt) of telomere repeat copy number (Ct telomere) to single control gene copy number (Ct GAPDH) in each sample. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the single control gene. Then the fold changes ($\Delta\Delta Ct$) relative to a reference value (mean dCT 1 day old group) was calculated. The PCR efficiency has been also taken into account. The amplification of telomeric regions was carried out using standard primers for vertebrates' telomeres: Tel1b (5′-CGG TTT GTT TGG GTT TGG GTT TGG GTT TGG GTT TGG GTT-3), Tel2b (5′-GGC TTG CCT TAC CCT TAC CCT TAC CCT TAC CCT TA CCC T-3) (Heidinger *et al.*, 2012; O'Callaghan and Fenech, 2011) and for GAPDH amplification were used own developed primers with following sequences: 5′- CAT CAA ATG GGC GGA TGC AG-3′ (GAPDH_F), 5′-GCA CCA GCA ATC CTT TTC CC-3′ (GAPDH_R_119). Each Real-time PCR reaction was performed in triplicate using 15 ng of extracted DNA, 0.2 uM of forward and reverse primers and RT-PCR kit with EvaGreen dye (Syntol, Russia) on CFX96 Detection System (Bio-Rad, USA). The PCR conditions were as follows: Initial activation of the Taq-DNA-Polymerase for 3 min at 95°C, followed by 30 cycles of 10 sec denaturation at 95°C, annealing for 15 sec at 60°C and extension for 1 min at 72°C.

For measuring of mitochondrial DNA (mtDNA) and nuclear DNA (nDNA) damage, the method based on

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endpoint quantitative PCR was used (Santos *et al.*, 2002). The principle of this technique is that lesions present in the DNA (oxidized bases, thymine dimers, etc.) block the progression of polymerase on the template and results in a decrease of PCR products. Analysis of long PCR products allows increasing the sensitivity of such assay substantially. The amplification of long PCR products was performed with own developed primers: MT_F (5′-ACC TTA GCC ATC ATC CCC CT-3′) and MT_R_100700 (5′-GGG TTG GGT TGT CGA CTG AA-3′) for mtDNA; GAPDH_F and GAPDH_R_10040 (5′-CTG TGG GGT TGG CAC AAA AG -3′) for nDNA. The length of amplicons made up 10070 bp and 10040, respectively. It is also important to note that GC content for both PCR products was similar: 46.7% for mtDNA amplicon and 47.9% for nDNA amplicon. But the part of coding DNA in mitochondrial PCR product is ten times higher. For long PCR we used 15 ng of DNA, 0.4 uM of forward and reverse primers and LongAmp Taq PCR Kit (New England Biolabs, UK). The PCR conditions were as follows: Initial denaturation for 1 min at 95°C, followed by 27-28 cycles of 10 sec denaturation at 95°C, annealing for 30 sec at 59°C and elongation for 9 min at 65°C, finally extension step for 15 min at 65°C. For mtDNA amplification, 27 cycles of PCR were used and 28 cycles - for nuclear. The quantity of PCR products was measured in three repetitions using fluorescent dyes analogically to DNA quantification. Although the initial amount of total DNA in each PCR sample was the same, the mtDNA/nDNA ratio could vary. Therefore, the addition normalization of template copy number was made. For standardization copy number, the RT-PCR of short amplicons with the same forward primers (MT_F, GAPDH_F) was done.

The reverse primers for short amplicons were MT_R_272 (5-CAG TGA TTA GGG AGG AGC CTT G-3) and GAPDH_R_119. According to data of RT-PCR the normalization coefficient was calculated as 1+E^Ct sample - mean Ct, where E = PCR efficiency. The efficiency of PCR was calculated using standard curves. paper is one part of the entire proceedings, not an independent document. Please do not revise any of the current designations.

Statistical Analysis

Statistical analysis was released using R-studio version 3.4.1. The Shapiro-Wilk test was used to check the normality of data. For data comparison, Student's T-test and Mann-Whitney U-test were done. For all possible pairwise comparisons, the Tukey method also was used. The correlation tests were done using Pearson and Spearman correlation coefficients. Differences were considered statistically significant at $p < 0.05$.

Results

The results of telomere length measurement by qPCR revealed the correlation between birds age and relative telomere length. dCt (Ct Tel-Ct GAPDH) values were as follows: 1 day old 7.5±0.4; 75 days old 6.6±0.7; 225 days old 5.5±0.5; 330-days old 4.9±0.7; 450 days old 4.3±0.9. Thus telomere length declined with age. The data of telomere length ratio (ΔΔCt values), which represent the variation of telomeres in 75-450 days old groups to mean value of control group (1 day old), are presented in Fig. 1. Such a figure more clearly shows the change coefficient in telomere length which make up: 0.88±0.08 for 75 days old, 0.73±0.07 for 225 days old, 0.66±0.1 for 330-days old and 0.58±0.12 for 450 days old.
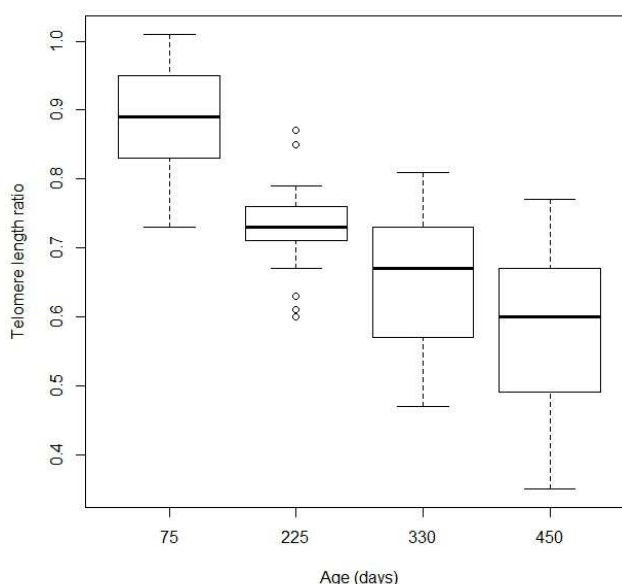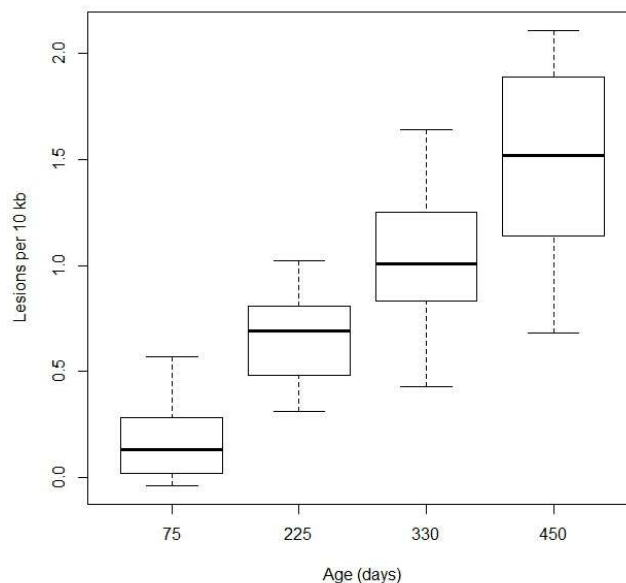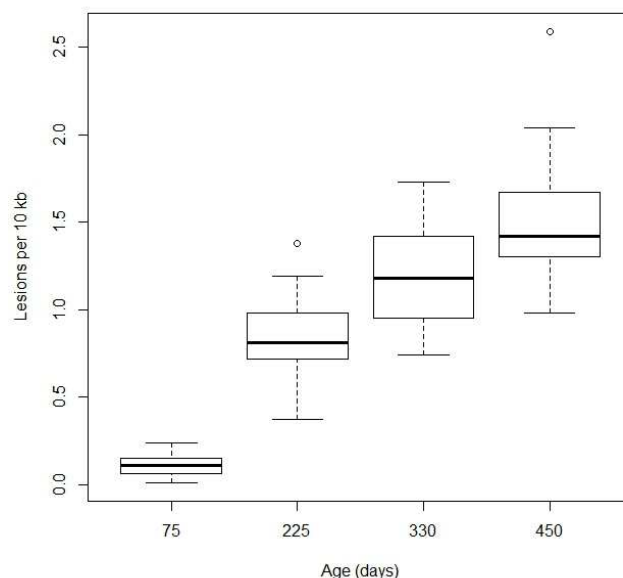

Fig. 1: The relationship between a ratio of telomere length of the control group mean value to age groups

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Fig. 2: The relative amount of lesions per 10 kb detected in the nuclear DNA (with normalization of mtDNA copy number)

Fig. 3: The relative amount of lesions per 10 kb detected in the mitochondrial DNA (with normalization of copy nDNA number)

Noticeable that, with increasing age, the relative standard deviation is rising from 9.2% (75, 255 days) to 14.8% (330 days) and 19.8% (450 days). The amount of nuclear and mitochondrial DNA damage of 75, 225, 330, 450 days old relative to control (mean value of 1 day old group) are presented in Fig. 2 and 3, respectively.

The relative quantity of lesions per 10 kb detected in the nuclear DNA were as follows: 0.17 ± 0.19 for 75 days old, 0.66 ± 0.21 for 225 days old, 1.02 ± 0.33 for 330-days old and 1.48 ± 0.45 for 450 days old and the quantity of lesions in mtDNA were as follows: 0.11 ± 0.06 for 75 days old, 0.84 ± 0.23 for 225 days old, 1.18 ± 0.3 for 330-days old and 1.49 ± 0.35 for 450 days old. According to received data, the amount of DNA lesions in both genomes (mitochondrial and nuclear) increased with age. Opposite to telomere length data, in both types of DNA lesions, the relative standard deviation was about equal in each age group except 75 days old group. All received data of telomere length, nDNA and mtDNA lesions relative to control are summarized in Table 2. The information about a difference of parameters between two consecutive age groups is also presented in Table 2.

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Table 2: Telomere length, nuclear and mitochondrial DNA lesions in chicken different age groups relative to control (1 day old group)

Age in days	Mean value	Standard deviation	The ratio value %*	Test type, P value
Relative telomere length				
75	0.88	0.08	-	-
225	0.73	0.07	16.9 %	T-test, 6.774e-09
330	0.66	0.10	10.6 %	T-test, 0.001999
450	0.58	0.12	11.5 %	T-test, 0.01387
Relative amount of lesions per 10 kb detected in the nuclear DNA				
75	0.17	0.19	-	-
225	0.66	0.21	390%	U-test, 6.856e-08
330	1.02	0.33	154%	U-test, 0.0001371
450	1.48	0.45	145%	U-test, 0.0008445
Relative amount of lesions per 10 kb detected in the mitochondrial DNA				
75	0.11	0.06		
225	0.84	0.23	770%	U-test, 1.398e-09
330	1.18	0.30	140%	U-test, 0.0001603
450	1.49	0.35	127%	U-test, 0.003082

* the ratio value was calculated as difference between two consecutive age groups in percentage

Discussion

Telomere length reduction is associated with wide range of genetic and environmental factors including sex, body size, reproduction, life stress, infection and chronic diseases (Paul, 2011). Consequently, investigations of human telomeres have controversial results in using telomere length as an accurate biological indicator of age (Mather *et al.*, 2011; Boonekamp *et al.*, 2013). However, in our study, there is a significant correlation between telomere length and hens age from 1 up to 450 days. The decrease of telomeres with age has been found in many other bird species studies: Sand martins, dunlins (Pauliny *et al.*, 2006), alpine swift (Criscuolo *et al.*, 2009), zebra finch (Heidinger *et al.*, 2012), European shags (Herborn *et al.*, 2014) and chicken (Kim *et al.*, 2011). However, we used a qPCR technique for telomere length measurements and the obtained data are comparable with Kim *et al.* (2011) research of chicken telomere length in 10-80 weeks age hens. In both studies, the most significant reduction of telomeres is observed in puberty period and a start of laying eggs stage 75-225 days in our research and 20-30 weeks. It should be noted that zebra finches have the most accelerated telomere shortening in the first year of life when they are engaging in reproduction (Heidinger *et al.*, 2012). However, comparing another study (Kim *et al.*, 2011) with current research, the lower telomere shortening ratio between age groups was discovered. In 75-225 days old groups we detected 17% decrease of telomere length as well as in 10-30 weeks age groups (Kim *et al.*, 2011) the difference was 38% and the reduction ratio of 225-330 days and 30-50 weeks made up 11 and 15%, respectively. Nevertheless, 330-450 days old and 50-70 weeks age groups had more similar telomere length decrease ratio 12 and 14%. Such variability may be explained by using different techniques (IQ FISH, qPCR) as well as various blood cells (lymphocytes, red blood cells). It should be noted that genetic factor such as breed could also play a role in telomere length, especially taking into account the difference in productivity of breeds (Sohn and Subramani, 2014). It is important to highlight that we first used 1 day old chicks as a control group, which in turn almost impossible be done in age studies on birds from wild populations for ethical reason.

The association of nuclear and mitochondrial DNA damage with age has also been detected. While telomere reduction in each subsequent age group was within 12-17%, the relative increase of DNA lesions was much higher (1.25-7.7 fold). The largest change, as in the case of a decrease in telomere length, is observed in hens of 75-225 days old. The increase in these age period was 7.7 fold for mtDNA and 3.9 for nDNA and the subsequent increase ratio was 1.25-1.5 fold.

Meanwhile, the association between age and telomere length, mtDNA, nDNA lesions was observed both correlation tests (Pearson and Spearman correlation coefficients) point out that the investigated parameters are changing independently. So age-dependent telomere length decrease is not associated with mtDNA and nDNA damage. As well as mtDNA and nDNA lesions accumulate independently, although, they have the similar trend to increase with age. It should be highlighted that the data obtained are actual for normal conditions. There are no doubts that in oxygen stress-induced conditions the increased mtDNA damage could be a reason for extra reactive oxygen species, which in turn have a significant impact on cellular senescence, including telomeres shortening (Passos *et al.*, 2007).

The most impressive result was gained while analyzing the relative amount of lesions in mtDNA and nDNA. Mean values of mtDNA and nDNA lesions in the

same age group were similar and have no significant difference, except 225 days old group wherein less damaged (on 21%) was nDNA. Age-dependent or induced DNA damage are 2-3 fold higher in mammalian mitochondrial DNA then in nuclear DNA (Wang *et al.*, 2010; Hamilton *et al.*, 2001). This argument is also confirmed on other organisms *Caenorhabditis elegans* (Meyer *et al.*, 2007), *Danio rerio* (Hunter *et al.*, 2010). Although our research data are opposite to other organisms' studies, the features of avian physiology could explain such a paradox. Compared with mammals the birds have much more efficient mitochondrial transport of electrons, which significantly (up to 10 times) reduces the generation of ROS (Montgomery *et al.*, 2012). So the avian mtDNA is less damaged by ROS, which results in total mtDNA lesions decrease. Summarizing, the assumption could be made - the chicken rate of mtDNA and nDNA lesions are relatively equal throughout their life period.

Chicken telomere length, mtDNA and nDNA lesions have significant dependents on age and can be used as an effective age-marker. The age-associated changes of chicken relative telomere length, mtDNA and nDNA lesions varying independently. Unlike mammal species, the chicken had similar DNA lesions rates in both mitochondrial and nuclear genomes for lifespan. The results obtained are of fundamental interest for aging processes, as well as could have an economic value for poultry farming.

Conclusion

Chicken telomere length, mtDNA and nDNA lesions have significant dependents on age and can be used as an effective age-marker. The age associated changes of chicken relative telomere length, mtDNA and nDNA lesions varying independently. Unlike many mammal species, chicken have similar DNA lesions rates in both mitochondrial and nuclear genomes for lifespan. The results obtained are of fundamental interest for aging processes, as well as could have an economic value for poultry farming.

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Author's Contributions

Maxim S. Makarenko and Aleksander V. Usatov: Designed and performed experiments and wrote the paper.

Lyubov V. Getmantseva and Vladimir A. Chistyakov: Developed analytical tools and analyzed data.

Aleksander I. Klimenko and Vyacheslav Vasilenko: Collected and analyzed data.

Ethics

This article is original and contains unpublished materials. The corresponding author confirms that all of the other authors have read and approved the manuscript and no ethical issues involved.

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