Age and heat stress as determinants of telomere length in a long-lived fish, the Siberian sturgeon.

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What is already known: Telomeres shorten with age in most endotherm species, but the picture in other vertebrate species and especially in fish remains less clear. In endotherms, telomeres shorten more rapidly when facing stressful environmental conditions, but this has never been tested in fish species.

What this study adds: We first review the results of the 14 studies investigating age-related changes in telomere length in fish species (Table 1) and then provide new data showing telomere shortening with age in a very long-lived fish, both at the intra- and at the inter-individual levels. In addition, we showed that a chronic sublethal heat stress could accelerate telomere erosion, suggesting that stressful environmental conditions could also accelerate telomere shortening in fish species.

Abstract

Telomeres shorten at each cell division due to the "end-replication problem", but also in response to oxidative stress. Consequently telomeres shorten with age in many endotherms, and this shortening is accelerated under stressful environmental conditions. Data in ectotherm vertebrates remain scarce so far, so our goal was to review existing data for fish, and to test the influence of age and stress on telomere length in a very long-lived fish, the Siberian sturgeon (*Acipenser baerii)*.

Our review of the literature revealed age-related telomere shortening in approximately half of the published studies. In the Siberian sturgeon, we found a significant telomere shortening with age, both at the intra-individual level using red blood cells (-12.5% in 16 months) and at the inter-individual level using cross-sectional samples of fin over an age-range of 8 years. We also found that heat stress (30°C) significantly reduced telomere length by 15.0% after only 1 month of exposure. Our results highlight that both age and stressful environmental conditions might be important determinants of telomere length in fish.

Keywords: fish, telomere, review, ageing, stress, *Acipenser baerii*

Introduction

Telomeres are specialized non-coding repeated DNA sequences ($TTAGGG_n$) located at the end of eukaryotic chromosomes playing an important role in the protection of genome integrity (De Lange et al. 2006). Telomeres shorten at each cell division due to the "end-replication problem", but they also shorten in response to oxidative stress (Zglinicki 2002). Consequently, telomeres shorten with time, unless being elongated by a specific enzyme called telomerase (De Lange et al. 2006). In most non-proliferative somatic tissues of adult mammals and birds (with a few exception), telomerase activity is low or undetectable (Gomes et al. 2010), and consequently telomeres usually shorten with age (*e.g.* Haussmann et al. 2003). A critically short telomere length has been shown to induce cell death or replicative senescence, and as a result of this it has been suggested that telomere shortening is an important cellular mechanism underpinning biological ageing (Monaghan and Haussmann 2006). The causality of such a link has however recently been questioned (Simons 2015). Yet, telomere length or telomere shortening rates have been found to predict survival or longevity in human (*e.g.* Bakaysa et al. 2007) and some bird species (see Stier et al. 2015 for a recent review). Additionally, telomere erosion rate has been associated with species longevity in mammals and birds, with long-lived species exhibiting less telomere loss with age than short-lived ones (Haussmann et al. 2003; Dantzer and Fletcher 2015).

Contrary to endotherms, we have little information about telomere dynamics in relation to age and environmental conditions in vertebrate ectotherms. Interestingly, telomerase activity is maintained even at adulthood in numerous tissues of ectotherm species (Gomes et al. 2010). In reptiles, we have some cross-sectional data showing either telomere maintenance (Hatase et al. 2008; Plot et al. 2012) or shortening (Bronikowski 2008; Xu et al. 2009) with age. At present, there is no robust information about age-related variation

67 in the length of amphibian telomeres. In fish, a few studies have looked at the influence of
68 age on telomere length, but no clear consensus has emerged so far. Our first objective was
69 thus to provide an exhaustive review of the literature in fish to better understand the link
70 between telomere dynamics, age and species longevity. Noting some specific gaps in the
71 literature, we were interested in obtaining data about age-related variation of telomere
72 length in a long-lived fish species (objective 2). Indeed, we only have information about
73 short- to medium-lived fish species to date, and patterns of telomere shortening with age
74 might differ between short and long-lived species, as already documented in endotherms
75 (Haussmann et al. 2003). Our biological model, the Siberian sturgeon (*Acipenser baerii*), is a
76 very long-lived fish with an average lifespan of 60 years (Pikitch et al. 2005). Using farmed
77 fish, we were able to obtain longitudinal data on telomere dynamics for a period of 16
78 months, and cross-sectional data on telomere length covering an age-range of 8 years. It is
79 worth noting here that longitudinal data (*i.e.* repeated sampling of a same individual over
80 time) is important to ascertain telomere dynamics with age, since an age-related decline in
81 telomere length could be masked in cross-sectional studies (*i.e.* comparing individuals of
82 different ages at a given occasion) by the selective disappearance of individuals with short
83 telomeres.

84 Although biomedical and ecological studies in endotherms have linked short
85 telomeres to stressful environmental conditions and impaired health status (*e.g.* Epel et al.
86 2004; Armanios and Blackburn 2012; Monaghan 2014; Asghar et al. 2015; Meillère et al.
87 2015; Stier et al. 2016), to our knowledge the impact of stressful environmental conditions
88 on telomere length has never been properly tested in fish. Water temperature is one critical
89 environmental parameter for both farmed and wild fish (Hart and Reynolds 2008). Heat
90 stress is a classic cause of stress in sturgeon, with high temperature being associated for

instance with reduced growth rate and increased juvenile mortality (Kappenman et al. 2009). Consequently, we investigated the impact of a chronic sublethal heat stress (*i.e.* 30°C for 1 month) on telomere length in juvenile sturgeons (objective 3), predicting that heat stress will be associated with a rapid rate of telomere shortening.

Material and Methods

Study animals and sampling procedures

All the fish used in this study were born in captivity, and came from the same hatchery (Ecloserie de Guyenne, France). They were raised under controlled conditions at 18°C until being at least three months old. In this paper, we used 3 batches of fish that were raised under different conditions after 3 months of age, but were fed with the same standard diet (*Efico sigma©*, Biomar, France). Within each batch, all the animals were kept in comparable conditions in terms of tank size and fish density. Juvenile fish (< 2 year old) were of undetermined sex, while adults were only females.

The first batch constituted of 22 juvenile fish has been kept in hatchery conditions until 19 months old. Fish were individually identified using PIT tags (Biolog-Tiny, Biolog-id, France). We collected two blood samples (≈ 100 µL) via caudal puncture, when the fish were 3 and 19 months old. Heparinised blood was immediately centrifuged (10min, 2500g, 4°C), and the resulting pellet of red blood cells (RBCs) was homogenized in 1mL of 100% ethanol before being stored at -80°C until further processing. This longitudinal sampling allowed us to investigate intra-individual variation in telomere length with age (objective 2).

The second batch was constituted of 121 fish divided in four age groups. The first age group (3 months old) was constituted of 26 juvenile fish from the hatchery. The remaining fish were maintained in outdoor tanks under natural conditions of temperature, and were

115 divided in three age groups (*i.e.* 3-4 years: N = 32; 5-6 years: N = 32; 7-8 years: N = 31).
116 Water temperature was unfortunately not monitored, which might potentially introduce
117 some bias in our analysis of age-related telomere shortening. However, exposing the fish to
118 natural variations of environmental parameters is likely to better reflect what happened in
119 the wild, especially compared to the well-controlled conditions experienced by fish kept
120 indoors such as in batch 1. One piece of pectoral fin was collected from each fish and
121 immediately placed in 100% ethanol before being stored at -80°C until further processing.
122 This extensive sampling allowed us to investigate inter-individual variation in telomere
123 length with age (objective 2).

124 The third batch was constituted of 36 fish being 5 months old at the beginning of the
125 experimental period. They were maintained under controlled conditions at 20°C before the
126 beginning of the experiment. They were randomly allocated to a control group (N =18)
127 maintained at 20°C or a heat stress group (N = 18) maintained at 30°C for 1 month. At the
128 end of the experimental period, a blood sample was collected, processed and stored as
129 detailed above for batch 1. This experiment allows us to investigate the impact of stressful
130 conditions on telomere length (objective 3). Fish care was conducted in accordance with the
131 institutional committee for animal care and use, and complied with French and European
132 regulations on animal welfare.

133 **Relative Telomere length measurement**

134 In the Siberian sturgeon, telomeric repeats ($TTAGGG_n$) have been localized only at the
135 end of chromosomes (*i.e.* true telomeres; Fontana et al. 1998), giving the opportunity to
136 measure relative telomere length using the qPCR method as described by Cawthon (2002).
137 Genomic DNA from RBCs and fins were respectively extracted using NucleoSpin 8 Blood Kit
138 (Macherey-Nagel, Germany) and DNeasy Blood and Tissue Kit (Qiagen, Germany) following

the manufacturer's recommendations. Real-time PCR amplifications were performed using the SYBRgreen technology on a LightCycler 480 (Roche, Germany). The reaction mix consisted of 5 µl of master mix (Roche, Germany), 1 µl of each primer (1 µM final), 1 µl of molecular grade water and 2 µl of DNA (20ng final). All assays were carried out in duplicate and we included a negative control and a calibrator on each plate in order to standardize runs with each other. Telomere primers were tel1: 5'-CGGTTTGTTTGGGTTTGGGTTTGGGTTTGGGTTTGGGTT-3' and tel2: 5'-GGCTTGCCTTACCCTTACCCTTACCCTTACCCTTACCCT-3'. The control gene was the β-actin as recently used in other fish species (Pauliny et al. 2015). β-actin primers were actin-forward: 5'-TATCCTGACCCTGAAGTACCCAATC-3' and actin-reverse: 5'-CACGCAGCTCATTGTAGAAGGTGTG-3'. PCR conditions for the two pairs of primers were 10 min at 95°C followed by 35 cycles of 1 min at 95°C and 1 min at 56°C. The specificity of the β-actin primers was assessed by migration on agarose gel of fifteen amplification products revealing one single band at the expected size, and through sequencing the amplicons of two individuals. Standard curves were generated and the amplification efficiencies were of 99.0 ± 1.3 % and 98.0 ± 1.1 % for telomere and β-actin amplifications respectively. A melting curve was calculated for each sample to control for non-specific amplification. Telomere length signal (T) is expressed relative to the β-actin signal (S). Final calculation of telomere length (T/S ratio) was done using the telomere and control gene real efficiencies of each run (Pfaffl 2001). Both the intra-plate variation based on T/S ratio of duplicates (CV = 5.5 ± 0.5%), and the inter-plate variation based on T/S ratio of the calibrator (CV = 5.9%) were low.

Statistics

We tested the effect of age on RBC telomere length at the intra-individual level (batch 1) using a Generalized Estimating Equation (GEE). We used fish ID as the individual factor and

age as the repeated effect. We tested the effect of age on fin telomere length at the inter-individual level (batch 2) using a General Linear Model (GLM) with age as the fixed factor. We used Bonferroni corrected post-hoc tests to investigate significant differences between the four age classes. Finally, we tested the effect of heat stress on RBC telomere length (batch 3) using a GLM with treatment as the fixed factor. Telomere data was log-transformed to achieve normality assumptions, but we decided to present raw data in the figures to avoid confusion due to negative log values. Means are always presented ± SE, statistical tests are always two-tailed, and p-values ≤ 0.05 were considered significant. Statistical tests were performed using SPSS 20.0.

Results

Literature review

We summarized the 14 available studies to date in Table 1. Nine of these studies found at least partial evidence for telomere shortening with age, nine of these studies found at least partial evidence for telomere maintenance with age, and only one study showed significant telomere lengthening (Table 1). Some studies found contrasted results depending on the type of tissue analyzed or the strain of fish used (see Table 1 for details).

Age effect in Siberian sturgeon

Using the longitudinal sampling of RBCs (batch 1), we found an intra-individual shortening of telomeres with age (GEE: $\chi^2 = 31.1$, $p < 0.001$; Figure 1A). Telomeres shortened on average of 12.5% during the 16 months of the study, but we found important inter-individual differences in the rate of telomere change (from -29.6% to +6.9%; Figure 1A).

Using the cross-sectional sampling of fins (batch 2), we found a significant effect of age on telomere length (GLM: $F = 40.2$, $p < 0.001$; Figure 1B). We found that telomeres of 3-

187 month-old fish were significantly longer than older age groups (all $p < 0.001$), and that fish
188 that were older than 7 years had significantly shorter telomeres than younger age groups (all
189 $p < 0.001$; Figure 1B). However, fish from the intermediate age groups (*i.e.* 3-4 *vs.* 5-6 years)
190 did not differ significantly in terms of telomere length ($p = 1.0$; Figure 1B). Yet, it is important
191 to note the pronounced inter-individual differences, for example, some individuals between
192 3 and 8 years old had telomere length resembling the range normally found in juvenile fish
193 (Figure 1B).

194 **Heat stress effect in Siberian sturgeon**

195 We found a significant effect of heat stress on telomere length (GLM: $F = 5.86$, $p =$
196 0.021), with fish exposed to 30°C presenting telomeres on average 15.0% shorter than
197 controls after only 1 month of exposure (Figure 2).

198

199 ## Discussion

200 The literature review that we conducted suggests that telomere shortening with age
201 occurs in fish species in approximately half of the studies published to date. Importantly,
202 only three studies used a longitudinal sampling to date, which limits our ability to definitively
203 form a conclusion about age-related telomere shortening, maintenance or lengthening in
204 most cases. Interestingly, the longevity of the species under investigation does not seem to
205 influence the likelihood of observing telomere shortening with age, which contrasts to what
206 is known in endotherms (Haussmann et al. 2003). As a remarkable example, the longest-
207 lived species (i.e. Siberian sturgeon) in our review exhibits an age-related decline in telomere
208 length, while the short-lived strain of the shortest-lived fish species does not (Table 1).
209 However, gathering precise information on telomere shortening rate (i.e. base pair loss per
210 unit of time) between species will be useful in the future to ascertain such ideas. According

211 to our review of the literature, it seems that the discrepancies between studies are more
212 likely linked to species-specific patterns rather than methodological considerations (i.e. type
213 of tissue, longitudinal *vs.* cross-sectional studies, method of telomere length measurement).

214 We found evidence of telomere shortening with age in a long-lived fish species, both
215 using longitudinal sampling of RBCs and cross-sectional sampling of fin samples. Considering
216 the exceptional longevity of this species, it might seem counterintuitive at a first glance that
217 we found evidence of telomere shortening with age, especially considering the limited time-
218 window of our study compared to the longevity of the species in the wild. Still, our results
219 agree with many others studies in fish, as shown in Table 1.

220 It is worth noting that we found marked individual differences in the rate of telomere
221 shortening (Figure 1A), but also in telomere length *per se* among age-matched individuals
222 (Figure 1B). Such heterogeneity might have multiple causes, such as individual differences in
223 physiological stress levels (*e.g.* glucocorticoids, oxidative stress), in telomerase expression
224 levels, or even in infectious status (see Asghar et al. 2015 for an example in birds). However
225 we have no information so far about the biological relevance of such heterogeneity in this
226 species; for instance in predicting survival as shown in human and birds (Bakaysa et al. 2007;
227 Stier et al. 2015), or even fecundity as shown recently in a short-lived fish species (Gao and
228 Munch 2015). Investigating potential relationships between telomere length and fecundity
229 will be of particular interest in the case of farmed sturgeon considering the economic value
230 of caviar.

231 Environmental stress is likely to be an important determinant of telomere shortening
232 in fish, since we found significantly shorter telomeres in sturgeons exposed to chronic heat
233 stress. Despite the relatively brief exposure to heat stress (*i.e.* 1 month), telomeres were
234 15.0% shorter in fish exposed to this treatment than in control fish. Rollings et al. (2014),

found no significant differences in telomere length between mosquitofish being raised at 20 or 30°C. However, while mosquitofish were well within their normal thermal range (*i.e.* 0-45°C; Pyke 2008), our Siberian sturgeons were slightly above their normal thermal range (*i.e.* 1-27°C; Williot 2002), possibly explaining this discrepancy. This suggests that sturgeons in our study were exposed to a substantial heat stress (as confirmed by an increased expression of heat shock protein 90; Simide et al. 2016), while mosquitofish in Rollings et al. (2014) were only exposed to a warmer temperature. According to our results, telomere length has the potential to be used as an indicator of chronic stress in sturgeons by fish farmers. However, gathering information about the impact of other stressors such as crowding or infection will be of tremendous importance in validating telomere length as an integrative marker of stress and welfare in fish. Finally, our results highlight the need to work with fish of known age and history for future studies looking at the impact of environmental conditions on telomere length.

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Author contributions

RS designed the study and conducted the experiments. AS contributed to the original idea of the study. FA and SG contributed to laboratory analyses. AS and RS analyzed the data. AS and RS wrote the manuscript. FA and SG commented on the manuscript.

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Table 1: **Review of the available studies investigating the relationships between telomere length and age in fish species**. We reported lifespan estimate for the study species and the tissue type(s) being used to measure telomere length in each study. Study monitoring is divided into cross-sectional (C) and longitudinal (L) approaches, method of telomere length measurement is indicated as quantitative PCR (qPCR), terminal restriction fragment (TRF) or quantitative fluorescence in situ hybridization (qFISH), and finally arrows describe telomere shortening (↘), maintenance (↔) or lengthening (↗) with advancing age.

Species		Lifespan estimate	Tissue type	Monitoring	Method	Age-related variation	Reference
Turquoise killfish	*Nothobranchius furzeri*	1.1[a]	Muscle, skin	C	qPCR, qFISH and TRF	↔ (short-lived strain) ↘ (long-lived strain)	Hartman et al. 2009
Atlantic silverside	*Menidia menidia*	2[a]	Brain, muscle	C	qPCR	↔	Gao & Munch 2015
Eastern mosquitofish	*Gambusia holbrooki*	(3)[a]	Muscle	C	qPCR	↘	Rollings et al. 2014
Japanese medaka	*Oryzias latipes*	5[a]	Brain, gonad, heart, intestine, kidney, liver, muscle, whole-body	C	TRF	↘ (except heart and brain)	Hatakeyama et al. 2008
Japanese medaka	*Oryzias latipes*	5[a]	Gill, Liver	C	TRF	↘	Gopalakrishnan et al. 2013
Coho salmon	*Oncorhynchus kisutch*	5[a]	Fin	**L**	qPCR	↔ (wild-type) ↘ (GH-transgenic)	Pauliny et al. 2015
Zebra fish	*Danio rerio*	5.5[a]	Brain, gill, heart, intestine, liver	C	TRF	↔	Lund et al. 2009
Zebra fish	*Danio rerio*	5.5[a]	Whole-body	C	TRF and qFISH	non-linear (↗↔↘)	Anchelin et al. 2011
Zebra fish	*Danio rerio*	5.5[a]	Fin	C	TRF	↘	Henriques et al. 2013
Brown trout	*Salmo trutta*	6[a]	Fin	**L**	qPCR	↔	Näslund et al. 2015
Port Jackson shark	*Heterodontus portusjacksoni*	(12)[a]	Gonad, muscle, red blood cells	C	qPCR and TRF	↔	Izzo 2010
European sea bass	*Dicentrarchus labrax*	15[a]	Red blood cells	C	TRF	↔	Horn et al. 2008
Blackhead seabream	*Acanthopagrus schlegelii*	15[b]	Muscle, red blood cells	C	TRF	Muscle: ↘[d] RBCs: ↗ (captive); ↔ (wild)	Tsui et al. 2005
Mangrove snapper	*Lutjanus argentimaculatus*	18[a]	Brain, muscle, red blood cells	C	TRF	↘	Tsui et al. 2005
Siberian sturgeon	*Acipenser baerii*	60[c]	Fin, red blood cells	C & **L**	qPCR	↘	This study

[a] Anage online database; [b] Y. Iwatsuki, pers. Com. 2009; [c] Pikitch et al. 2005. Lifespan estimates given between brackets are those available for the closest related species. [d] when captive and wild fish are analyzed together following data extraction from figure ($R^2 = 0.20$, $p = 0.014$).

Figure legends

Figure 1: **Relationships between relative telomere length and age**: (A) at the intra-individual level using telomere length of RBCs (N = 22, $p < 0.001$), (B) at the inter-individual level using telomere length measured in fin samples (N = 121, $p < 0.001$). Means are presented ± SE and letters indicate significant differences.

Figure 2: **Effect of 1-month exposure to heat stress (30°C) on RBCs telomere length of 6 months old Siberian sturgeons** (N = 36, $p = 0.021$). Means are presented ± SE and * indicates significant difference between experimental groups.

Figure1

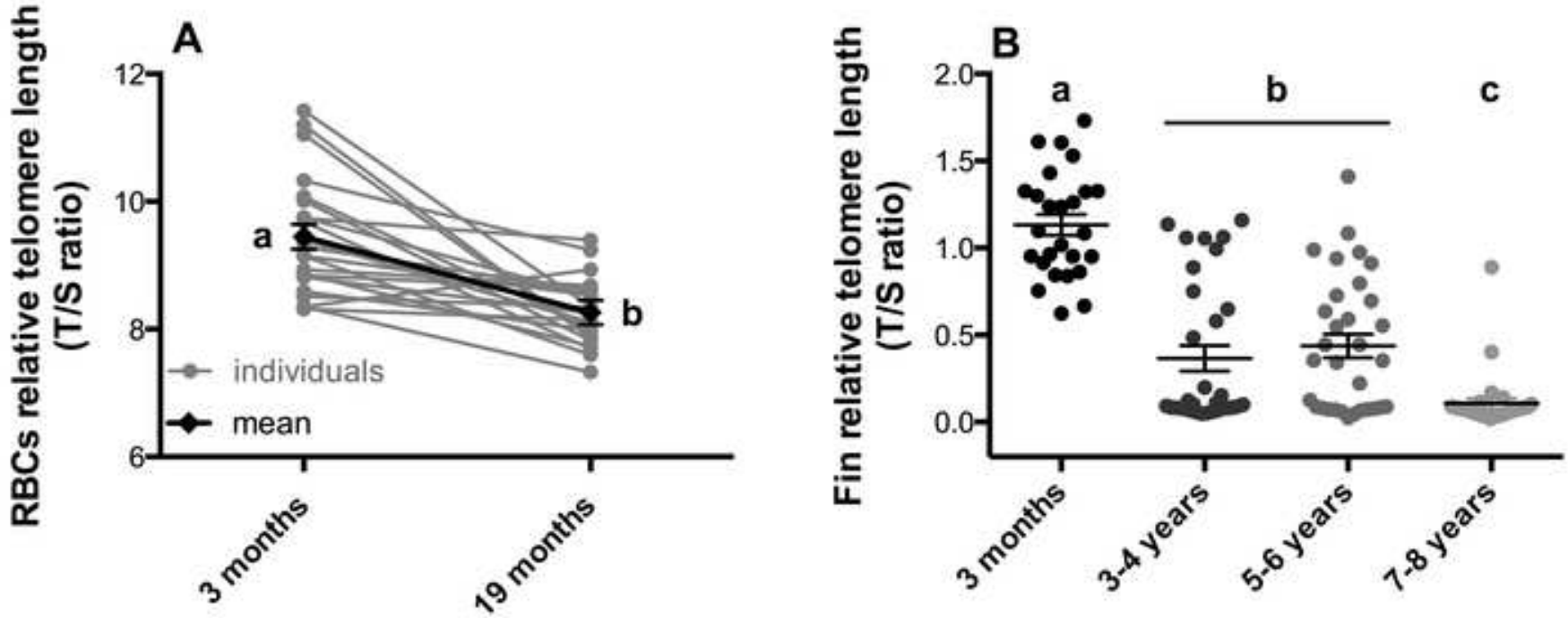
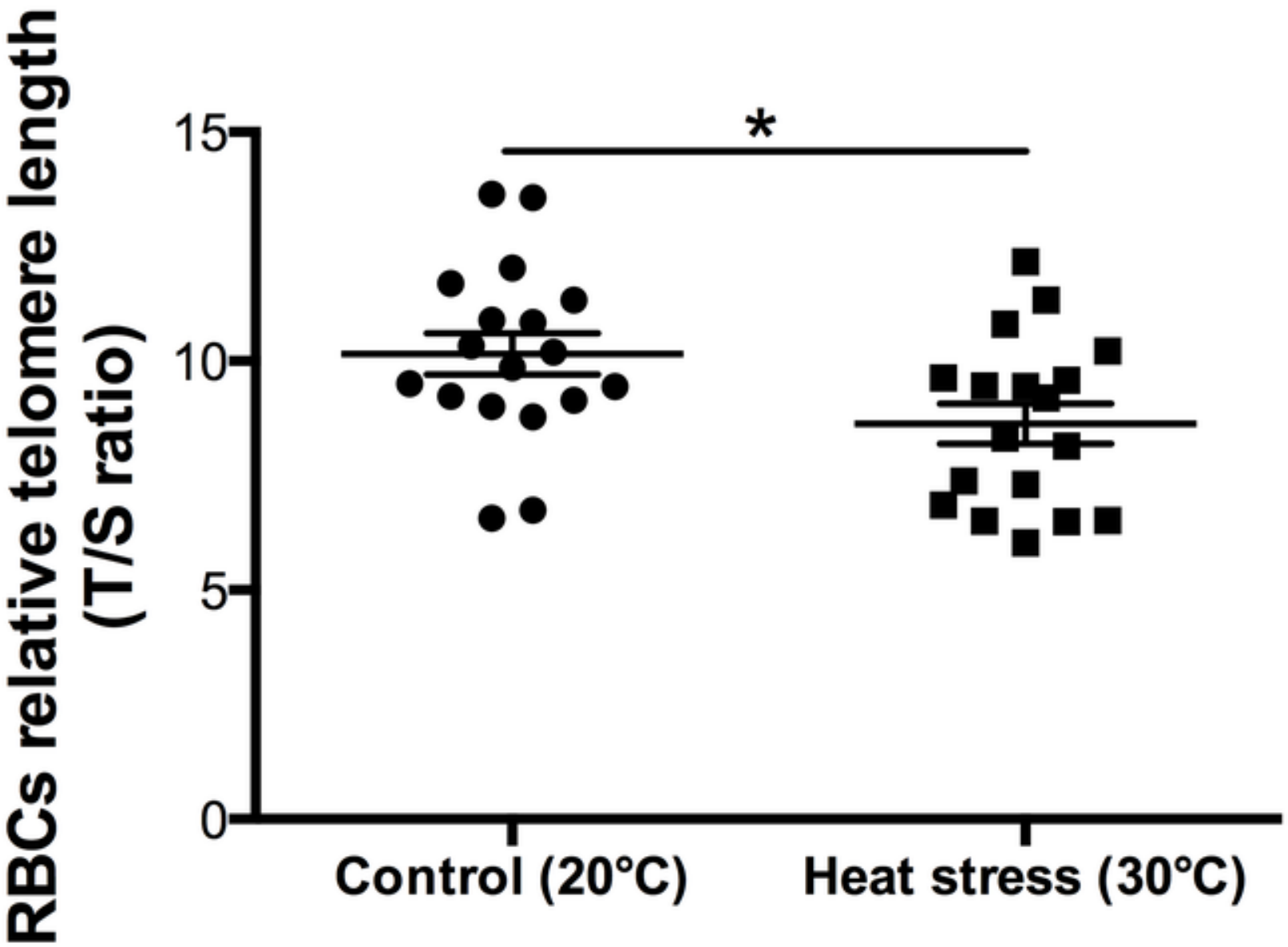

A
RBCs relative telomere length (T/S ratio)
12
10
8
6
a
b
individuals
mean
3 months
19 months
B
Fin relative telomere length (T/S ratio)
2.0
1.5
1.0
0.5
0.0
a
b
c
3 months
3-4 years
5-6 years
7-8 years

Figure2


RBCs relative telomere length
(T/S ratio)
15
10
5
0
*
Control (20°C)
Heat stress (30°C)