Age at maturation has sex and temperature specific effects on telomere length in a fish

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ABSTRACT

Telomeres are highly conserved nucleoprotein structures which protect genome integrity. The length of telomeres is influenced by both genetic and environmental factors, but relatively little is known about how different hereditary and environmental factors interact in determining telomere length. We manipulated growth rates and timing of maturation by exposing full-sib nine-spined sticklebacks (*Pungitius pungitius*) to two different temperature treatments and quantified the effects of temperature treatments, sex, timing of maturation, growth rate and family (genetic influences) on telomere length. We did not find the overall effect of temperature treatment on the relative telomere length. However, we found that variation in telomere length was related to timing of maturation in a sex- and temperature-dependent manner. Telomere length was negatively related to age at maturation in elevated temperature and early maturing males and females differed in teleomere length. Variation in growth rate did not explain any variation in telomere length. The broad sense heritability (h^2) of telomere length was estimated at $h^2 = 0.31\text{-}0.47$, suggesting predominance of environmental over genetic determinants of telomere length variability. This study provides the first evidence that age at maturation together with factors associated with it are influencing telomere length in an ectotherm. Future studies are encouraged to identify the extent to which these results can be replicated in other ectotherms.

Keywords: aging, heritability, *Pungitius pungitius*, telomere, temperature

INTRODUCTION

Telomeres are nucleoprotein structures whose main function is to protect genome integrity (Blackburn 2000). Telomeres shorten with every cell division which eventually leads to cellular senescence and various associated pathologies (Blasco 2005). The process of telomere attrition can be accelerated by an array of stressors, and thus an individual's telomere length may be indicative of its exposure to stress and/or its stress resistance (von Zglinicki 2002; Epel et al. 2004; Mizutani et al. 2013). Unsurprisingly, individual variation in telomere length has been linked to variation in various phenotypic attributes, including survival probability (Bakaysa et al. 2007; Monaghan 2010; Heidinger et al. 2012; Angelier et al. 2013). While telomere shortening can be to some extent restored by telomerase enzyme, the process of telomere erosion is usually faster than their elongation (Barrett and Richardson 2011). Furthermore, the process of telomere restoration may be costly either due to elevated risk of pathologies or because of diversion of resources which could otherwise be allocated to other vital life-history functions such as reproduction (Campisi 2005; Monaghan and Haussmann 2006). In fact, when extrinsic mortality is high, investments into the costly maintenance of telomere length maybe wasteful (i.e. "disposable soma theory"; Kirkwood 1977; Kirkwood and Rose 1991). Therefore, whether the benefits of telomere length restoration offset its costs may be highly context dependent, may differ among sexes and among individuals who have experienced different growth histories.

Sex is one of the most important drivers of intraspecific life-history variation (Rice 1984; Slatkin 1984), and telomere length has been repeatedly found to differ between sexes mirroring sex-specific differences in the lifespan (Barrett and Richardson 2011). While the proximate causes for this remains unclear, it is conceivable that sex differences in life histories may also drive variation in telomere dynamics. This possibility is especially interesting in case of sexually dimorphic species in which the

two sexes may often differ markedly in optimal values of important life history traits (Roff 1993). For example, the optimal age at maturation may be very different for males and females. In ectotherms, such as fish, early maturation is associated with a smaller size, which especially in case of females translates to reduced fecundity (Roff 1993; Shimada et al. 2011), whereas male reproductive success is not, or only weakly so, affected by age at maturation (Adams and Huntingford 1997; Uusi-Heikkilä et al. 2011). Furthermore, experimental studies modulating growth conditions found that high growth rate is associated with early maturation (Kuparinen et al. 2011) and reduced longevity (Lee et al. 2013). Therefore, individuals and sexes with different maturation schedules likely differ in their investment into different life-history traits. Consequently, studies of sex-bias in telomere shortening in species with sex-specific differences in life-history strategies can be particularly rewarding (Barrett and Richardson 2011). However, up to date, only a limited number of studies have studied sex differences in telomere shortening in species where the sexes differ conspicuously in their life histories (Foote et al. 2010; Gopalakrishnan et al. 2013; Rollings et al. 2014; Gao and Munch 2015; Peterson et al. 2015).

As most other quantitative traits, variation in telomere length is known to be influenced both by environmental and genetic factors (Broer et al. 2013; Asghar et al. 2015). Most of what is known about heritability of telomere length comes from human studies where it ranges from a moderate 36% to as high as 90% (Bischoff et al. 2005; Baird 2008; Broer et al. 2013). Since traits closely related to fitness are expected to have low heritability (Price and Schluter 1991; Houle 1992; Merilä and Sheldon 1999), the high heritability recovered in human studies could indicate that variation in telomere length is not closely associated with variation in fitness, or that the heritabilities have been overestimated for a reason or another. Evolutionarily more informative insights on the genetic basis of telomere length should be obtainable from studies of non-model organisms (Kappei and Londoño-Vallejo 2008; Monaghan 2010), and such information has been accumulating recently (Horn et al. 2011; Olsson et al.

2011; Voillemot et al. 2012; Reichert et al. 2015; Asghar et al. 2015; Atema et al. 2015; Becker et al. 2015). However, most of these studies have been conducted with endothermic birds, and to best of our knowledge, only one study has focused on inheritance of telomere length variation in ectothermic vertebrate (Olsson et al. 2011). However, the study of Olsson *et al*. (2011) is problematic in the sense that it utilized parent-offspring regressions for heritability estimation, in spite of the fact that one of the critical assumptions underlying the usage of this approach is that the comparable trait is measured in parents and offspring. Clearly, if telomeres of parents and offspring are not quantified at the same age and telomere length changes with age, parent-offspring regression may give biased estimates of heritability.

The aim of this study was to assess whether variation in growth rate and age at maturation were associated with variation in telomere length in nine-spined sticklebacks (*Pungitius pungitius*), and whether these associations were sex-specific. We predicted that i) variation in telomere length will be negatively associated with variation in growth rate, and that ii) mature individuals will have shorter telomeres than immature individuals. The latter may be expected either because of the slower growth, and thus also lower cell division rate, of immature individuals and/or the fact that maturation itself is energetically costly. We do not exclude a possibility that if the trade-off between growth and telomere length arises due to competition for energy and resources it may be infact masked by resource acquisition capacities of individuals. Also, because female maturation on average requires more resources than male maturation (Hayward and Gillooly 2011), we expected that iii) mature females would have shorter telomeres than mature males.

In order to test these predictions, we used data from an experiment where growth rate and timing of maturation were manipulated by exposing individually reared full-sib individuals from the same nine-spined stickleback families to two ecologically relevant temperatures (Kuparinen et al. 2011). In

addition, leveraging the relatively large set of full-sib families in the data, we evaluated the relative importance of genetic *vs* environmental influences (i.e. heritability) in determining variation in telomere length.

MATERIAL AND METHODS

Fish sampling and rearing

Adult nine-spined sticklebacks were caught with seine nets in 2008 in the Baltic Sea (60°10′ N; 25°00′ E) and transported to laboratory facilities at the University of Helsinki (Finland). Nine full-sib families were created by artificial fertilization as described in (Kuparinen et al. 2011). Fertilized eggs were incubated at 17°C and fry were photographed within few hours after hatching to measure hatching size. Shortly after hatching, individuals ($N = 400$) were randomly assigned to one of the two temperature treatments (14 °C and 17°C) and two replicates per each family and each treatment so that families were equally represented. Fish were reared individually in 1.4L tanks which were separated by opaque plastic sheets. Tanks were arranged in four racks (Allentown Zebrafish Rack System, Aquaneering, San Diego, USA) housing 100 individuals per rack. Each rack had a separate water circulation system where water was filtered by physical, chemical, biological and UV filters. Light conditions during the entire experiment were set to 24 hour light to mimic high-latitude summer conditions, as well as to enhance growth and development. Individuals were initially fed with live *Artemia,* but gradually food was changed to *Chironomidae* larvae. When individuals were 17 days old, weekly size measurements were initiated to follow individual growth trajectories. These size measurements continued until the fish were 15 weeks old (i.e. until 115 days of age) and were obtained by taking a digital photograph from which body length (from the tip of the nose to the end of the tail base) was measured using a software TpsDig 1.4 (Rohlf 2002).

Age at maturation was closely monitored during the entire duration of the experiment. Upon their maturation, male nine-spined sticklebacks develop distinctive nuptial coloration, and were thus considered mature when first signs of such coloration were observed. Females in this species do not change their coloration visibly when mature, and thus, their maturation was monitored by observing their reproductive status by gently squeezing visually gravid females (twice a day) so as to see if eggs were ready to be released. Mainly due to mortality in early growth stages a substantial proportion (34.75 % or 139 individuals out of 400) of individuals were lost. The experiment was terminated when the fish were122 days old and when approximately half of the remaining individuals had matured (*N* [matured individuals/total number of individuals alive] = 109/261). Individuals were euthanized with an overdose of MS-222 (tricainemethanesulfonate) and their sex was confirmed or identified (in case of immature individuals) by visual examination of the gonads. Brains were dissected out and stored at -80°C until further analyses.

Telomere assay

Telomere length was determined as the ratio between the amount of telomeric repeats and that of a reference sequence by using quantitative polymerase chain reaction (qPCR; Cawthon 2002). Quantified this way, the relative telomere length (RTL) corresponds to an average telomere length across all chromosomes (Cawthon 2002). This approach to quantify telomere length has been successfully applied in many ecological studies (e.g., Olsson et al. 2011; Heidinger et al. 2012; Plot et al. 2012). The known shortcoming of this method is that if present, qPCR amplifies also interstitial telomeric repeats generating noise for inter-individual comparisons (Foote et al. 2013; Nussey et al. 2014). However, interstitial telomeric repeats have not been detected in nine-spined sticklebacks (Ocalewicz et al. 2011). Telomeric repeats were amplified using universal primers developed by Cawthon (2002):

Tel1b: CGGTTTGTTTGGGTTTGGGTTTGGGTTTGGGTTTGGGTT;

Tel2b: GGCTTGCCTTACCCTTACCCTTACCCTTACCCTTACCCT.

The zinc finger protein 1 gene (*zic1*) was used as a reference sequence. A partial sequence of zic1from *Pungitius pungitus* was obtained from GenBank (Accession Number:AB445219) and primers amplifying a fragment of this gene were designed using Primer 3 software (Untergasser et al. 2012):

PuZic1Fw.: CAACAGGCGAAGTCACAGAG ,

PuZic1Rev.: CGTGGGAGCTGTGGTTTATT.

For the telomere assay, genomic DNA was extracted from brain tissue using QIAamp Fast DNA Tissue kit (QIAGEN) following manufacturer's instructions. The integrity and purity of the extracted DNA was checked by agarose gel electrophoresis and NanoDrop 2000 (ThermoFisher), respectively. Only visually intact samples with a $A_{260/280}$ ratio higher than 1.7 (mean ± SD = 1.85 ± 0.11) were accepted for further analyses. The qPCR reactions were run separately for telomere and reference sequence amplification in a Bio-Rad X1000 real time thermal cycler (BIO-RAD) in 384-well microplates (BIO-RAD). Each reaction mix included iTaq ^{TM} DNA polymerase, dNTPs, $MgCl_2$ and fluorescein-SYBR found in iQ^{TM} SYBR® Green qPCR mix (BIO-RAD), plus primers and 5ng of DNA template. For telomere reactions 100nM ofTel1b and 300 nM of Tel2b were used, while for *zic1* (reference sequence) 200nM for both forward and reverse primers were used. All plates included serial doubling dilutions (from 1.25 ng/well to 20 ng/well) of a standard sample, which was made by pooling equal quantities of DNA from five randomly picked individuals. Each plate also included one extra standard sample and a no-template control where DNA volume was substituted with water. All reactions were carried out in triplicate. Individuals were randomly distributed in plates so that all families were proportionally represented on each of the reaction plate. The qPCR thermal cycling protocol for telomere fragment amplification started with an initial denaturation step at 95^{o}C for 5 min followed by 21 cycle of 95°C for 30s, 55°C for 15s and 72°C for 30s. Conditions for *zic1* fragment amplification were as follows:

95°C for 5min, and 40 cycles of 95°C for 20 s, 59°C for 30s and 72°C for 30s. As the final stage of both protocols melt-curves were generated by slowly (0.1°C/s) increasing temperature from 70 to 95°C. LinRegPCR software was used to determine amplicon specific window of linearity, Cq (threshold cycle when amplification signal crosses the background level) and individual well efficiencies (Ramakers et al. 2003). Plates were standardized for the between plate variation using GenEx 6 software (MultiD, Göteborg). Coefficients of variation (CV) between replicates of the same sample were calculated in percents, and replicates with CV > 5% were excluded (2 out of 660 values [0.3%] for *zic1* reactions, and 9 values out of 660 [1.36%] for telomeres). Resulting mean within replicate repeatability calculated as intraclass correlation coefficient (ICC) and CV were high both for *zic1*(ICC_{zic1} = 0.90, CI [0.88-0.92], CV_{zic1}= 0.71%)and telomere primer reactions (ICC_{TL} = 0.87, CI [0.83-0.89], CV_{TL} = 1.73%). Finally, relative telomere length (RTL) was calculated for 213 samples using delta CT method as:

$$RTL = 2^{(C_t^{TL} - C_t^{zic1})_{standard} - (C_t^{TL} - C_t^{zic1})_{focal}} \quad (1)$$

where C_t – critical cycle for TL and reference gene (*zic1*) respectively. To account for potential biases caused by measurement error we have also calculated all combinations of RTL using three replicates of telomere and *zic1*Cq values. Intra-individual repeatability of telomere length calculated in this way was significant (ICC = 0.68, CI [0.64-0.72]). Mean estimated RTL-value was 1.10 (±0.44 SD, range [0.25-3.86]). We have also used a more common approach of calculating mean values of the replicate Cq values and thus obtaining only one RTL estimate per individual. Mean estimated RTL-value using this approach was 1.16 (±0.46 SD, range [0.47-2.43]). One sample more than three standard deviations away from the mean was removed as an outlier.

Statistical analyses

198 Individual growth curve parameters were obtained using the von Bertalanffy growth curve equation
199 (von Bertalanffy 1938) and fitting the equation with body size measurements at given age (l) through
200 non-linear least-squares regression (2):

201 $$l(t) = L_\infty - (L_\infty - L_0)e^{-kt} \quad (2)$$

202 yielding three measures portraying individual growth: k –the intrinsic growth coefficient; L_0 – size at
203 t=0 and L_∞-asymptotic length (Kuparinen et al. 2011). The von Bertalanffy equation described the data
204 very well with the mean goodness of fit estimate of 0.99 (±0.004 SD). Since growth coefficient and
205 asymptotic size were highly correlated (r_p= 0.76, P <0.01) only asymptotic length (L_∞) was included in
206 further statistical analyses. Age at maturation is not a growth curve parameter while it is still
207 proportional to asymptotic size (r_p=0.53, P <0.01).
208 In order to test whether telomere length is associated with growth and maturation in a sex specific
209 manner, we constructed a linear mixed effect (LME) model in which RTL was the response variable,
210 and the fixed explanatory variables were sex, maturation status (matured or unmatured), asymptotic
211 size (L_∞) and temperature treatment. Initial models included two-way interactions between these main
212 explanatory variables. We also included hatching size as covariate to control for possible differences in
213 *in ovo* conditions, which may affect telomere length. Family identity was included as a random factor
214 and the model was fitted with maximum likelihood. Model selection progressed in a step-wise manner
215 by performing likelihood ratio test and excluding non-significant terms from the model (Table1).

216 As the second analysis step, we constructed an identical model to the one described above, with the
217 difference that the binary maturation status was substituted with a continuous age at maturation (Table
218 2). Since only 109 out of the 261 surviving individuals reached maturity before the end of the
219 experiment, the sample size in this analysis was substantially lower than in the former. The initial
220 model was reduced using the backward step-wise elimination procedure as described above. Residuals

of the final model adhered to the assumption of normality and variance inflation factors (VIF) were lower than 2.2 for all variables included in the model (Dormann et al. 2013). In all analyses, telomere length was log-transformed before analyses to assure normal distribution of residuals.

Heritability of telomere length

To estimate broad sense heritability (h^2) of telomere length, we used an animal model approach as implemented in the MCMCglmm package in R (Hadfield 2010). Four MCMCglmm models were run which differed in random and fixed effect structure. Firstly, a model where only a binary maturation status was included as a fixed effect was run. Secondly, a model which included variables and interactions obtained during LME model (final model) selection using a continuous age at maturation variable was run. These models included either 'family' term as a random effect or both 'family' and 'individual' random effects. The later random effect structure was used for models were multiple RTL estimates were calculated and allows to account for measurement error arising due to small variation between technical replicates. We used an inverse Wishart prior for the variance component estimation. Models were run for 1 000 000 (models with error term) and 5 000 000 (models without error term) iterations discarding the first 100 000 runs as burn-in in both cases, and there after sampling every 500th iteration. This allowed to obtain 1800-9800 samples from the posterior distribution. Heritability was calculated as genetic variance divided by the total variance and credible intervals for heritability estimates were given as highest posterior density intervals (HPDI*).*

All statistical analyses were performed in R 2.13.0 (R Core Team 2011).

RESULTS

We did not find any main effect of experimental temperature on telomere length (likelihood ratio (*LR)* = 0.045, *df = 202, P* = 0.83). Likewise, a linear mixed effect model using maturation status (mature *vs*. immature) as an explanatory variable did not reveal any significant main or interactions effects on variation in telomere length (Table 1). Thus, telomere length variation among individuals was not explained by their maturation status (*LR* = 0.021, *df* = 200*, P* = 0.885), sex (*LR* = 0.208, *df* = 201*, P* = 0.648), temperature (*LR* = 0.005, *df* = 199*, P* = 0.945) or individual's asymptotic length (*LR* = 1.365, *df* = 203, *P* = 0.243; Table 1). Similarly, hatching size did not explain any variation in telomere length (Table 1).

After restricting analysis to individuals which matured and including individual's age at maturation as a covariate, different results emerged. There was a significant interaction between an individual's sex and its age at maturation (*LR* = 5.818, *df* = 69*, P* = 0.016; Table 2, Fig. 1) and between age at maturation and temperature treatment (*LR* = 7.000, *df* = 69, *P* = 0.008; Table 2, Fig. 2). However, telomere length was not related to L_∞ (*LR* = 0.033, *df* = 67, *P* = 0.855; Table 2) or hatching size (*LR* = 1.121, *df* = 68, *P* = 0.290; Table 2).

For models where a binary maturation status was used as an explanatory variable, broad sense heritability for telomere length was 0.47 (HPDI: 0.17-0.91) when measurement error was taken into account and 0.38 (HPDI: 0.12 - 0.90) when a mean of replicates was used to calculate RTL estimates. Heritability of telomere length was respectively 0.37 (HPDI: 0.09-0.91) and 0.31 (HPDI: 0.05 - 0.97) when a continuous age at maturation was included as an explanatory variable.

DISCUSSION

The most important finding of this study is the experimental demonstration that individual variation in telomere length among sexually mature fish is influenced by rearing temperature, sex, as well as by the age at which individuals matured. The effect of maturation timing on telomere length was modulated by sex and temperature treatment so that telomere length in females, but not in males, decreased with increasing age at maturation. Similarly, telomere length decreased with increasing age at maturation for individuals exposed to the high temperature treatment whereas not for individuals in the low temperature treatment. However, we did not find any evidence to support the expectation (cf. Geiger et al. 2012; Lee et al. 2013) that individuals with higher growth rates would have shorter telomeres). Also, we did not find that temperature treatment would have had an overall effect on telomere length. Consistent with observed environmental influences on telomere length, we also discovered that although variation in telomere length was partially influenced by genetic effects ($h^2 \approx 0.31$-47), much of the variation appears to be of an environmental origin. The relatively low heritability of telomere length in nine-spined sticklebacks stands in contrasts to much higher estimates from some earlier studies (Horn et al. 2011; Olsson et al. 2011; Voillemot et al. 2012; Broer et al. 2013; Reichert et al. 2015; Asghar et al. 2015; Atema et al. 2015; Becker et al. 2015).

Interactive effects between sex and age at maturation

Our initial expectation that maturation status (cf. matured *vs* immature) would explain variation in telomere length was not supported by the data. A follow-up analysis using age at maturation as a continuous predictor and ignoring immature individuals revealed that age at maturation had a negative effect on telomere length. However, this effect was different between the two sexes. One possible explanation for this difference is that costs of maturation are sex-specific. As in many other fish species, delayed maturation in female nine-spined sticklebacks is related to a higher reproductive output (Herczeg et al. 2010; Shikano and Merilä 2011). Although we did not analyze reproductive

287 investment in this study, increased reproductive investment is shown to be associated with shorter
288 telomeres in other fish (Gao and Munch 2015) and in birds (Bauch et al. 2013; Schultner et al.
289 2013).This may be because reproduction and particularly egg production is energetically costly, and
290 increases oxidative stress (Wang et al. 2001; Bertrand et al. 2005), which in turn accelerates telomere
291 shortening (von Zglinicki 2002).Thus, late maturing large females may be investing resources heavily
292 into egg production and have fewer resources left for self-maintenance such as, for example,
293 scavenging of reactive oxygen species which results in shorter telomeres. In contrast, since male
294 maturation (i.e. sperm production) requires substantially less energy than female maturation (Hayward
295 and Gillooly 2011), males may be left with sufficient resources for self-maintenance to avoid telomere
296 shortening.

297 Estrogen deficiency can inhibit telomerase activity (Kyo et al. 1999; Bayne et al. 2008),which is
298 normally active in all fish tissues throughout their life (Hatakeyama et al. 2008; Lund et al. 2009).
299 Similarly, estrogens may lower oxidative stress (Behl et al. 1997; Aviv 2006; Razmara et al. 2007) and
300 thereby work against telomere shorting. These considerations lead to prediction that males and late
301 maturing females should have on average shorter telomeres than early maturing females (Vihko and
302 Apter 1984; Emaus et al. 2008; Gopalakrishnan et al. 2013). This is what we indeed observed in nine-
303 spined sticklebacks in this study. Also data from medaka (*Oryziaslatipes*) supports this line of
304 reasoning: estrogen and telomerase activity peak at sexual maturation time in females, which is also
305 time when the greatest difference in telomere length is observed between sexes (Gopalakrishnan et al.
306 2013).This provides an alternative, but not mutually exclusive, explanation for the observation that
307 especially female telomere length is negatively related to age at maturation.

308 *Interactive effects between temperature and maturation*

309 Ectotherm metabolism, growth and to some extent also maturation is modulated by environmental
310 temperature (Angilletta 2009). In sticklebacks, higher temperatures increase growth and reduce
311 survival probability (Kuparinen et al. 2011; Lee et al. 2013). Temperature affects both maturation and
312 growth rate in females, while only growth rate is influenced by temperature in nine-spined stickleback
313 males (Kuparinen et al. 2011). In light of these findings, we were surprised not to find any direct
314 association between telomere length and asymptotic length or telomere length and an overall effect of
315 temperature treatment (Rollings et al. 2014). One possible explanation for the lack of expected
316 associations is *ad libitum* feeding regime which might have provided fish with enough energy to both
317 growth and self maintenance thereby masking an expected trade-off. This would also mean that the
318 lack of an overall temperature effect on telomere length may not be universal across contexts and may
319 depend on individual's ability to acquire resources. This possibly could translate into a positive
320 correlation between life-history traits in some individuals (Hamel e al. 2009). However, alternatively
321 and not exclusively, it may also be that individual differences in life-history strategies (e.g. relative
322 investment to growth and reproduction) could lead to differences in telomere length which arise at
323 certain life-history stages. In support for this expectation we observed that association between age at
324 maturation and telomere length differed between individual in the two temperature treatments (Fig.
325 2).This indicates that temperature may influence telomere length through maturation rather than growth
326 rate *per se*. However, much of the temperature effect appeared to be driven by the presence of early
327 maturing males with long telomeres in the high temperature treatment, suggesting that the temperature
328 effect is also influenced by sex. However, three-way interaction between sex, temperature and age at
329 maturation was never significant (results not shown).

330 *Heritability of telomere length*

331 Previous studies in non-model vertebrates have found that heritability of telomere length varies from
332 3.8 to 99 %, although some of these estimates were not significantly different from zero(Horn et al.
333 2011; Olsson et al. 2011; Voillemot et al. 2012; Reichert et al. 2015; Asghar et al. 2015; Atema et al.
334 2015; Becker et al. 2015). The majority of these studies were conducted on wild or captive birds (Horn
335 et al. 2011; Voillemot et al. 2012; Reichert et al. 2015; Asghar et al. 2015; Atema et al. 2015; Becker et
336 al. 2015), and only one of them involved an ectotherm, the sand lizard (*Lacertaagilis*; Olsson et al.
337 2011). To the best of our knowledge, our study is the second one to report heritability of telomere
338 length for an ectothermic vertebrate, and the first one for a fish species. Our estimate ($h^2 \approx 40\%$) is
339 fairly low, especially in the view that it is a broad-sense, rather than a narrow-sense estimate. Hence,
340 the true heritability is likely to be even lower than those estimated. This is because our estimate(s) may
341 include common environment and non-additive genetic contributions. Yet, the low heritability suggests
342 that environmental, rather than genetic, sources of variation are likely to explain most of the variance in
343 telomere length in fish.

344 It is tempting to speculate that the relatively low heritability of telomere length reported in this study –
345 in contrast to those of several other studies of natural vertebrate populations (Table 3) – might be
346 related to the fact that most other studies have been conducted in endothermic, rather than in
347 ectothermic (but see: Olsson et al., 2011) vertebrates. However, methods used for telomere length and
348 heritability estimation should be also considered (Becker et al. 2015). Many earlier estimates telomere
349 length heritability were obtained with parent-offspring regression, which assumes that the same trait is
350 measured at both generations (Lynch & Walsh 1998). However, telomere length does not always
351 exhibit a linear relationship with age (Hatakeyama et al. 2016). Therefore measurements on parents and
352 offspring might not be comparable, leading to biased heritability estimates. Similarly, full-sib estimates
353 of heritability can be problematic since they do not control for common environment or non-additive

genetic effects, and hence may provide upward biased estimate of heritability. However, upward-bias cannot explain the low heritability of telomere length found in this study: if our estimates were upward-biased, this would mean that the actual heritability of telomere length would be even lower than now estimated. With the accumulating information on estimates for heritability of telomere length across the taxa a meta-analysis would be especially rewarding and would allow to see what factors may be behind the observed discrepancies in telomere length heritability estimates. We also note that accounting for measurement error in telomere length estimates lead to change in heritability estimates, suggesting that part of the variation in heritability estimates across the studies might be explainable by differences how accurately the length of telomere lengths were estimated.

Limitations of the study

We utilized qPCR to estimate relative telomere length using the brain tissue. While qPCR has been successfully used in a large number of studies (Voillemot et al. 2012; Heidinger et al. 2012; Becker et al. 2015) and have undeniable advantages over telomere restriction fragment analysis, namely it is high-throughput (Nussey et al. 2014), concerns have been raised about possible biases caused by presence of interstitial telomericrepeats (Foote et al. 2013). This problem should not be an issue in this study because nine-spine sticklebacks are unlikely to have telomeric repeats positioned inside chromosomes (Ocalewicz et al. 2011). Further, qPCR approach may have relatively highmeasurement error if not optimized properly. Even a well-optimized assay yielding low CV's and high repeatability between technical replicates may lead to considerable measurement error. In this study we have taken this possibility into account by using two approaches to treat technical replicates and calculate heritability of telomere length. By using the two approaches we have obtained moderately low estimates of telomere heritability.

Another potential concern is that we have analyzed telomere length in brain tissue. Cell turn-over in neural tissue is usually lower than that in blood or liver, and since cell turn-over rate is related to telomere length (Sekoguchi et al. 2007), this might have influenced the inference. If the neural tissue we used had ceased its growth before sampling, then telomere lengths measured from such tissues could reflect some innate differences between individuals. If so, this could indicate that late maturing females and late maturing individuals in high temperature treatment had shorter telomeres because they were of a lower quality, and not because the maturation or treatment had causal influence on telomere length. This is not unthinkable because telomere length is indeed used as an indicator for individuals' phenotypic quality and is related to early life-conditions (Aviv 2006; Heidinger et al. 2012; Bauch et al. 2013). Nonetheless, the use of brain tissue is unlikely to be problem in our study for several reasons. First, telomere length and rate of telomere loss have been found to correlate significantly between different tissue types (Hatakeyama et al. 2008; Daniali et al. 2013; Gao and Munch 2015). Second, in contrast to vertebrates with determinate growth (Hastings et al. 2001), growth and neurogenesis in fish continues throughout their lives (Ganz and Brand 2016). Thus, the brain is capable for plastic responses to environmental influences even in adulthood (Park et al. 2012; Herczeg et al. 2015). In line with this finding, telomerase is active throughout a fish life in all tissues including brain potentially facilitating neural cell proliferation longer than it would be expected for mammals (Klapper et al. 1998; Hatakeyama et al. 2008). Lastly, we included hatching size in the models in order to control for possible initial quality related differences between individuals. While hatching size was positively correlated with size at maturation ($p=0.01$), it did not explain any variation in telomere length. Further risk of initial quality differences were decreased due to random assignment of subjects across the treatments.

In conclusion, the results show that variation in telomere length in nine-spined sticklebacks is influenced both by genetic and environmental factors, and that the latter is a greater source for the observed variability. While sex, age at maturation and temperature treatment all explained significant amount of variation in telomere length, their influence emerged due to interactive, rather than simple isolated effects of each factor. In particular, the results indicate that timing of maturation and factors influencing it may be connected to intra-specific variation in fish telomere length.

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Table 1. Linear mixed effect (LME) model selection for variables explaining variation in relative telomere length where a binary maturation status variable was included in the model.

Explanatory variable[a]	**Parameter estimate ±SE**	***df***	**Likelihood ratio (*P*)**[b]
Intercept	0.061 ± 0.064	204	
L_∞	0.004 ±0.004	203	1.365 (0.243)
Hatching size	0.070 ±0.128	202	0.299 (0.585)
Sex (male)	−0.022 ± 0.049	201	0.208 (0.648)
Maturation status (M; 1)	−0.008 ± 0.058	200	0.021 (0.885)
Temperature (T; 17°C)	0.003 ± 0.047	199	0.005 (0.945)
M × L_∞	−0.006 ± 0.007	198	0.603 (0.437)
Sex × M	−0.112 ± 0.110	197	1.057 (0.304)
L_∞× T	0.006 ± 0.007	196	0.782 (0.377)
Sex × T	0.059 ± 0.098	195	0.374 (0.541)
Sex × L_∞	−0.002 ± 0.009	194	0.082 (0.774)
M × T	0.009 ± 0.101	193	0.009 (0.925)

Abbreviations: L_∞-asymptotic length, SE-standard error, *df* - degrees of freedom.

[a] Sex = female, TEMP=14°C, MAT = 0 (immature) are included in the intercept and considered as references

[b]Likelihood ratio tests were conducted and p-values obtained by comparing models with and without the term

Table 2 Final model (in **bold**) and linear mixed effect (LME) model selection for variables explaining relative telomere length. A continuous age at maturation variable was included in the model.

Explanatory variable[a]	Parameter estimate ±SE	*df*	Likelihood ratio (*P*)[b]
Intercept	**0.795 ±0.666**	**69**	
Sex (male)	**−1.743 ±0.667**	**69**	**9.328 (0.009)**
Age at maturation (AM)	**−0.006 ±0.006**	**69**	**11.950 (0.008)**
Temperaturetreatment (T; 17°C)	**1.360 ±0.557**	**69**	**7.819 (0.020)**
Sex × AM	**0.015 ±0.006**	**69**	**5.818 (0.016)**
AM × T	**−0.014 ±0.005**	**69**	**7.000 (0.008)**
Hatching size	0.215 ±0.212	68	1.121 (0.290)
L_∞	−0.001 ±0.007	67	0.0334 (0.855)
AM × L_∞	0.001 ±0.000	66	2.0416 (0.153)
Sex × L_∞	0.027 ±0.019	65	2.483 (0.115)
L_∞× T	0.006 ±0.012	64	0.241 (0.624)
Sex × T	−0.072 ±0.233	63	0.111 (0.740)

Abbreviations: L_∞-, asymptotic length, SE-standard error, df-degrees of freedom

[a] Sex =female and TEMP=14°C are included in the intercept and considered as a references

[b]Likelihood ratio tests were conducted and p-values obtained by comparing models with and without the given term

Table 3 Synopsis of the published heritability estimates of telomere length from non-model organisms

Taxon	Species	h^2 (SE/CI)	Method for h^2 estimation	Telomere measure (method)	$n_{families}$	$n_{individuals}$	Reference
Teleostei	Nine-spined stickleback (*Pungitius pungitius*)	0.47 (0.17-0.91) 0.37 (0.09-0.91) 0.38 (0.12 - 0.90) 0.31 (0.05 - 0.97)	FS (Anim)	RTL (qPCR)	9	83; 213	current study
Reptilia	Sand lizard (*Lacertaagilis*)	0.52; 1.23	PO	Average TL (TRF)	40 (daughter-dam) 80 (son-sire)	80♀ 110♂	(Olsson et al. 2011)
Aves	Kakapo (*Strigopshabroptilus*)	0.84	PO	Average TL (TRF)	29	29 offspring 29 mothers	(Horn et al. 2011)
	Collared flycatcher (*Ficedulaalbicollis*)	0.09; 0.18*	FS	RTL (qPCR)	74	359	(Voillemot et al. 2012)
	White-throated dippers (*Cincluscinclus*)	0.038 (0.069)	Anim	RTL (qPCR)	NA	177	(Becker et al. 2015)
	Great reed warbler (*Acrocephalusarundinaceus*)	0.35 (0.07); 0.48 (0.12)	Anim	RTL (qPCR)	46	193	(Asghar et al. 2015)
	King penguin (*Aptenodytespatagonicus*)	0.2 (0.1); 0.3 (0.1)	PO	RTL (qPCR)	53	53 offspring 106 parents	(Reichert et al. 2015)
	Zebra finch (*Taeniopygiaguttata*)	0.99 (0.87-1)	Anim	Average TL (TRF)	73	125	(Atema et al. 2015)

*- value reported to be different from originally published in the paper (Becker et al. 2015)

631 Anim = animal model, FS = full-sib analysis; PO = parent offspring regression; TRF = telomere
632 restriction fragment analysis; qPCR = quantitative PCR analysis; RTL = relative telomere length; TL =
633 telomere length; SE = standard error; CI = confidence interval.

634

635 **Figure legends**

636 **Fig. 1** Relative telomere length (RTL) as a function of age at maturation in male and female nine-
637 spined sticklebacks. RTL decreases with postponed maturation in female sticklebacks (f, open circles,
638 dashed line), while males show little response in RTL with maturation schedule (m, black dots, solid
639 line)

640

641 **Fig. 2** Relative telomere length (RTL) as a function of age at maturation in high (17°C, open circles,
642 dashed line) and low (14°C, black dots, solid line) temperature treatments in nine-spined sticklebacks

643

644 **Fig. 3** Mean and variation in residual relative telomere length (RTL) between nine-spined stickleback
645 families (F4 to F12) included into the study. In the box plot, 25 to 75 percentiles of the data are
646 enclosed by the box and a median is marked with a horizontal line. Whiskers show range of the data

647

Fig. 1

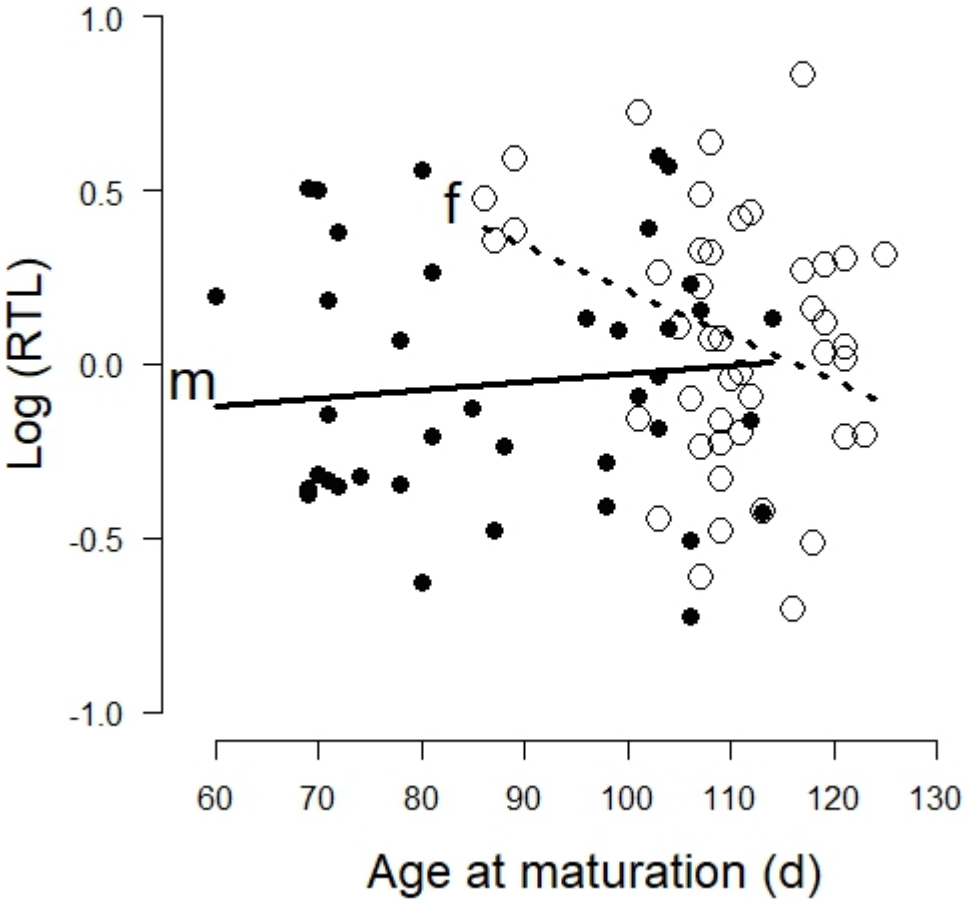

1.0
0.5
0.0
-0.5
-1.0
Log (RTL)
f
m
60
70
80
90
100
110
120
130
Age at maturation (d)

Fig. 2

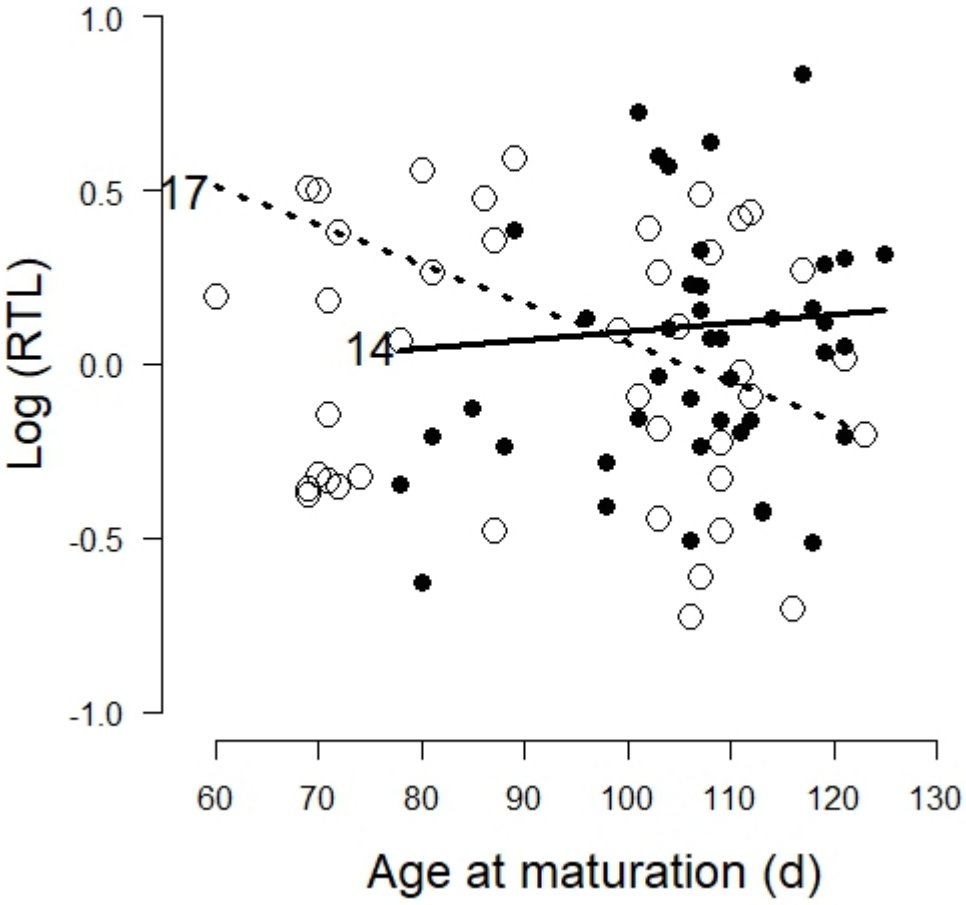
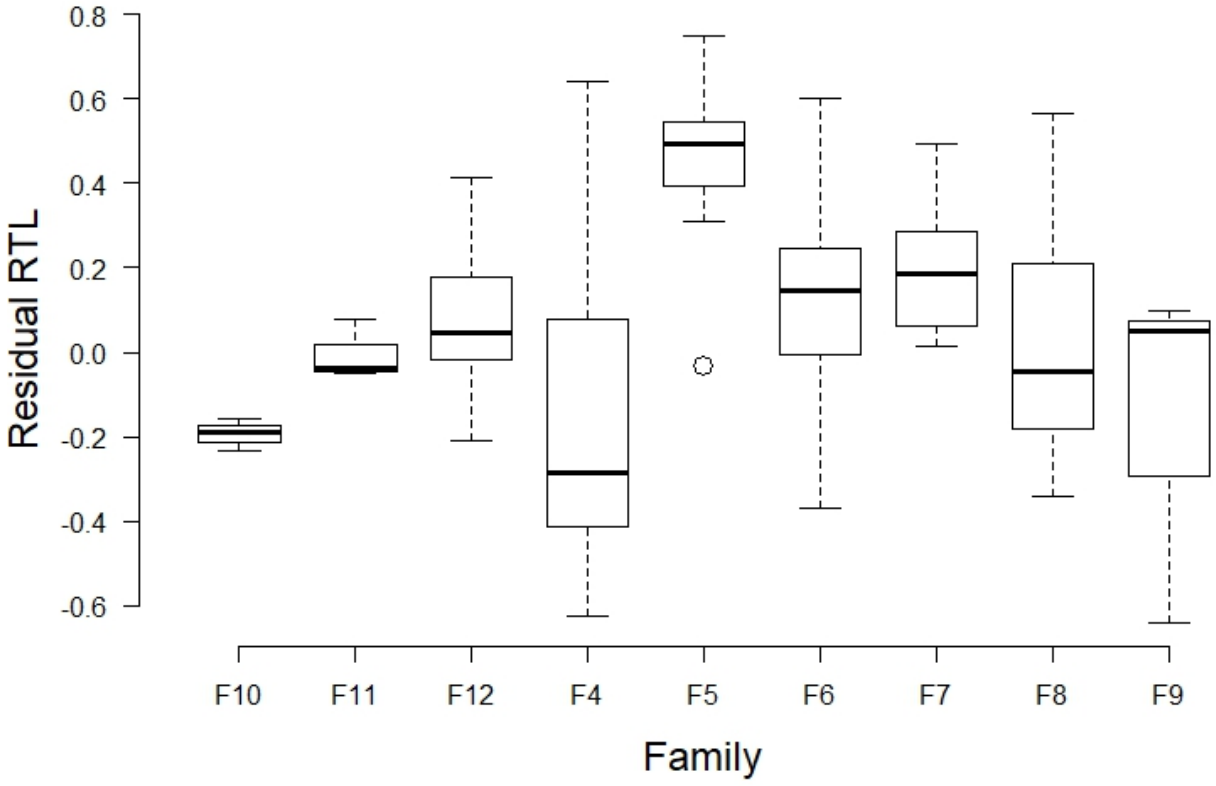

1.0
0.5
0.0
-0.5
-1.0
Log (RTL)
17
14
60
70
80
90
100
110
120
130
Age at maturation (d)

Fig. 3


Residual RTL
0.8
0.6
0.4
0.2
0.0
-0.2
-0.4
-0.6
F10
F11
F12
F4
F5
F6
F7
F8
F9
Family