Telomere length, individual quality and fitness in female European starlings (*Sturnus vulgaris*) during breeding Criscuolo, F. [1], Fowler, M.F. [1,3], Fuhrer, V.A. [1], Zahn, S[1]. and Williams T.D. [2]

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Ratios have particular statistical properties. For instance, ratio's normal distribution highly depends on the coefficient of variation of the numerator and denominator variables, which is most case is assumed to be equal (Atchley, Gaskins & Anderson 1976). Several solutions have been previously proposed to resolve this potential problem, among which replace the use of ratio by an analysis of covariance of both concerned variables (Packard & Boardman 1988). Here we proposed to test the use of linear regression residuals between the amplification signals of the telomere sequence and the control gene to assess relative telomere length from qPCR amplification data.

As shown in the ESM figures, the residuals of Cq-Tel / Cq-RAG1 are following a normal distribution without any need of log-transformation. Positive residuals mean that Cq Tel are high for a given Cq-RAG1 value: as high Cq values correspond to short telomeres (*i.e.* the sequence needs more time to be amplified), positive residuals characterized short telomeres, after controlling for the DNA quantity actually present in the well (*i.e.* the Cq-RAG1 value). Running the models with the residuals leads to the same results as those presented in the main text of the paper. The loss of telomere length in the LQ females even reaches significance at P = 0,050 (see Table 3 below). In the light of these results, it appears to us that, depending on the significance and the r² of the linear regression between the Cq values of the telomere sequence and of the control gene, using the residuals seems to us as robust as the usual way of dealing with qPCR amplification data. We do not state here that relying on the T/S ratio to evaluate qPCR output of telomere amplifications (Pfaffl 2001) is spurious. In fact, ratios may not conduct to false statistical conclusions, particularly when the coefficient of variation of the numerator is higher than the one from the numerator (Anderson & Lydic 1977), which shall be generally the case with telomere qPCR. Rather, we suggest to further test residuals utilization when

analyzing telomere amplification results, to better assess within which limits of normal distribution and of coefficients of variation of raw qPCR data, residuals may be more appropriate to use than the T/S ratio.

1. Q-Q plots of log-transformed telomere length (T/S ratio) and of Cq-Tel / Cq-RAG1 residuals.

Q-Q Plot of Log10-transformed telomere length (T/S ratio)

Q-Q Plot of Cq-Tel / Cq-RAG1 residuals
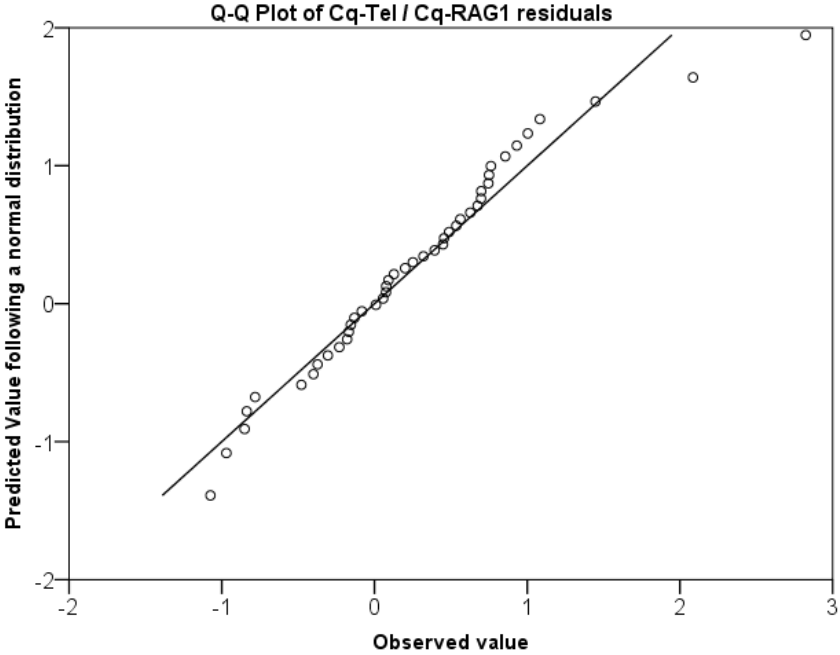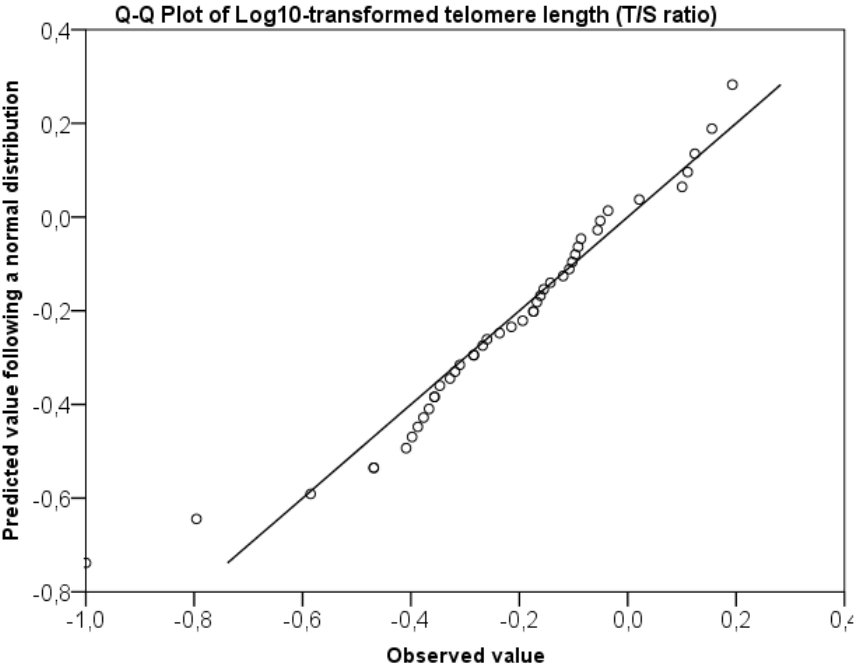
2. Results of the linear regression between Cq Tel and Cq RAG1 values (number of cycles of qPCR amplification for each sequence) measured in adult female starlings. R = 0,474, r^2 = 0,225. Residuals followed a normal distribution, Kolmogorov-Smirnov test, z = 0,544; P = 0,929.

Response variable: *Residuals Cq Tel – Cq RAG1*	Estimates	D.F	T	P
Intercept	-4.622 ± 4.503	1, 53	-1,026	0,310
Cq – RAG1	0.764 ± 0.197	1, 53	3.880	**<0.001**
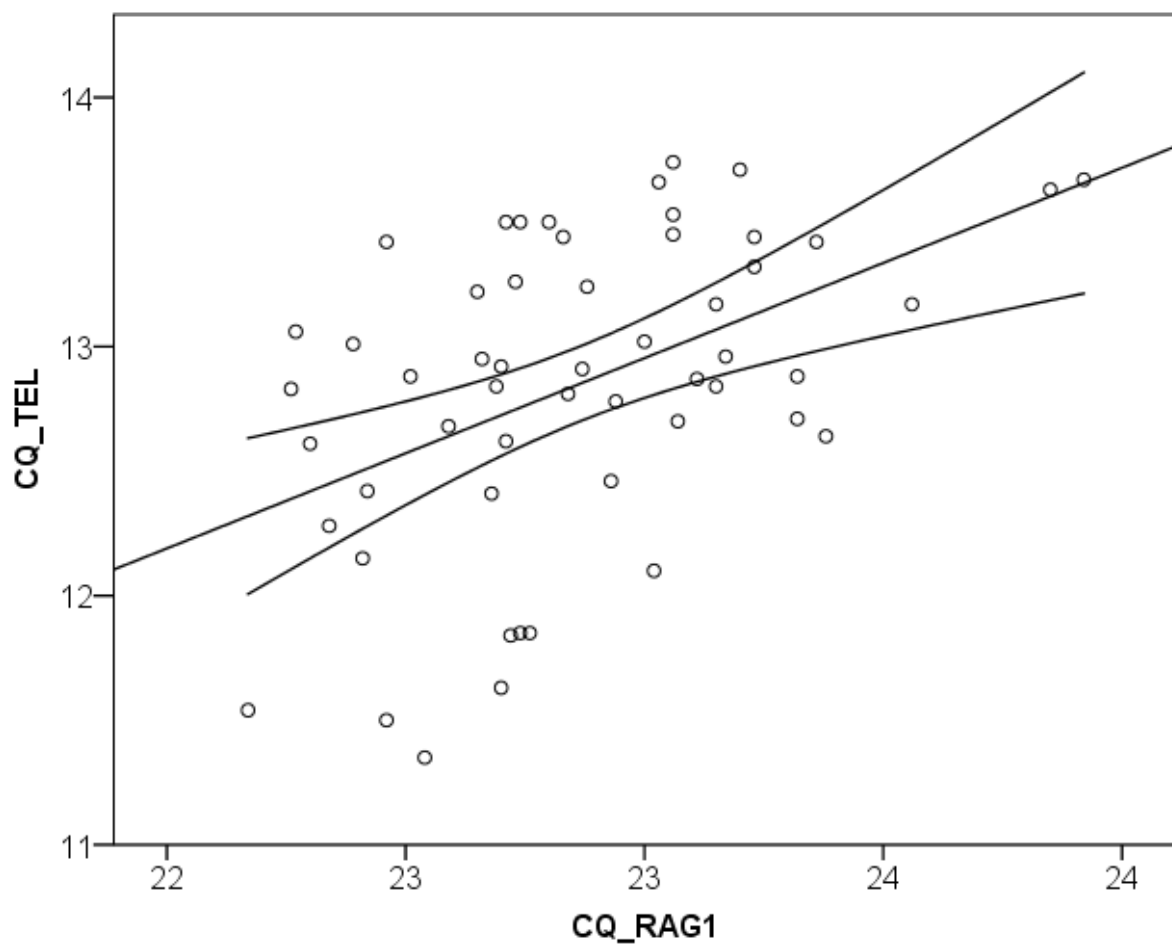
3. Table 1. Results of a Mixed Model with residuals of Cq Tel – Cq RAG1 as a response variable (relative telomere length), with individual quality and breeding stages as fixed factors and female identity as random factor. HQ = High Quality, MQ = Medium Quality, LQ = Low Quality. Post-hoc comparisons were done using Bonferroni corrections, among groups during the incubation and chick rearing stages, and within groups between incubation and chick rearing stages. Significant P values are indicated in bold. Residuals of each models followed a normal distribution (checked using Kolmogorov-Smirnov test and QQ plot).

Response variable: *Residuals Cq Tel – Cq RAG1*	Estimates	D.F	F	P
Intercept	0.042 ± 0.156	1, 27	16.689	**<0.001**
Individual Quality		2, 27	10.791	**0.005**
MQ females vs. HQ females	-0.428 ± 0.301			0.310
MQ females vs. LQ females	-0.809 ± 0.258			**0.005**
HQ females vs. LQ females	-0.382 ± 0.365			0.310
Breeding stage (Chick rearing vs. Incubation)	-0;042 ± 0.071	1, 27	1.922	0.166
Individual Quality x Breeding stage	1.524 ± 0.317	2, 27	7.457	**0.024**
During Incubation				
MQ females vs. HQ females	-0.714 ± 0.543			1.000
MQ females vs. LQ females	-0.204 ± 0.342			1.000
HQ females vs. LQ females	0.509 ± 0.603			1.000
During Chick rearing				
MQ females vs. HQ females	-0.141 ± 0.222			1.000
MQ females vs. LQ females	-1.414 ± 0.390			**0.001**
HQ females vs. LQ females	-1.272 ± 0.385			**0.012**
Between Incubation & Chick rearing				
Among HQ females	-0.443 ± 0.521			1.000
Among MQ females	-0.129 ± 0.234			1.000
Among LQ females	-1.338 ± 0.467			**0.050**

4. Table 3. Results of General Linear Models (with a logistic distribution for return rate and 2nd brood initiation) testing for relationships between residuals of Cq Tel – Cq RAG1 as a response variable (relative telomere length) of adult starling females and: (A) current effort of reproduction, (B) future prospects of reproduction during the same breeding season (2013, 2nd brood), (C) future prospects of reproduction during the next breeding season (2014), (D) total reproduction success over 2013 and 2014. In B and C models, first brood size (in 2013 and 2014) and first year total fledging number (in 2013) were included as a covariates to control for the initial brood or first year breeding costs. Adult telomere length was measured during the chick rearing period of the first brood. Significant results are indicated in bold.

A. Current breeding effort (2013)				
Response variable: ***Brood size at day 17***	Estimates	D.F	F	P
Intercept	3.642 ± 0.342	1, 19	113.156	**<0.001**
Residuals Cq Tel – Cq RAG1	-1.052 ± 0.450	1, 19	5.466	**0.030**
Response variable: ***chick provisioning rate***	Estimates	D.F	F	P
Intercept	4.971 ± 0.551	1, 17	9.021	**<0.001**
Residuals Cq Tel – Cq RAG1	-1.555 ± 0.715	1, 17	2.176	**0.044**
1st brood size	0.161 ± 0.361	1, 17	0.446	0.661
B. Same year breeding prospects (2013)				
Response variable: ***Initiation of a 2nd brood***	Estimates	D.F	F	P
Intercept	0.595 ± 1.300	1, 17	0.457	0.647
Residuals Cq Tel – Cq RAG1	-0.632 ± 0.793	1, 17	-0.797	0.425
1st brood size	-0.019 ± 0.335	1, 17	-0.057	0.955
Response variable: ***2nd brood size at fledging***	Estimates	D.F	F	P
Intercept	0.105 ± 0.942	1, 17	0.111	0.913
Residuals Cq Tel – Cq RAG1	0.336 ± 0.562	1, 17	0.597	0.559
1st brood size	0.149 ± 0.259	1, 17	0.606	0.553
c. Next year breeding prospects (2014)				
Response variable: ***adult female return rate***	Estimates	D.F	F	P
Intercept	-3.200 ± 1.494	1, 17	-2.143	**0.032**
Residuals Cq Tel – Cq RAG1	0.281 ± 0.925	1, 17	0.304	0.761
1st year fledging number	0.491 ± 0.299	1, 17	1.642	0.101
Response variable: ***2nd year, 1st brood size at fledging***	Estimates	D.F	F	P
Intercept	-0.529 ± 1.099	1, 17	-0.481	0.637
Residuals Cq Tel – Cq RAG1	0.399 ± 0.737	1, 17	0.541	0.596
1st year fledging number	0.446 ± 0.231	1, 17	1.931	0.071

Response variable: **2nd year, initiation of a 2nd brood**	Estimates	D.F	F	P
Intercept	0.595 ± 1.300	1, 17	0.457	0.647
Residuals Cq Tel – Cq RAG1	-0.631 ± 0.793	1, 17	-0.797	0.425
1st year fledging number	-0.019± 0.335	1, 17	-0.057	0.955
Response variable: **2nd year, 2nd brood size at fledging**	Estimates	D.F	F	P
Intercept	0.505 ± 0.338	1, 17	1.492	0.156
Residuals Cq Tel – Cq RAG1	-0.315 ± 0.227	1, 17	-1.386	0.186
1st year fledging number	-0.123 ± 0.078	1, 17	-1.563	0.139
2nd year, 1st brood fledging number	0.251 ± 0.076	1,17	3.301	**0.005**
Response variable: **2nd year total fledging number**	Estimates	D.F	F	P
Intercept	-0.157 ± 1.410	1, 17	-0.111	0.913
Residuals Cq Tel – Cq RAG1	0.185 ± 0.949	1, 17	0.195	0.848
1st year fledging number	0.436 ± 0.297	1, 17	1.467	0.162
D. Total breeding success (2013 and 2014) Response variable: **Total number of fledging over 2 years**	Estimates	D.F	F	P
Intercept	5.769 ± 1.070	1, 17	5.392	**<0.001**
Residuals Cq Tel – Cq RAG1	-1.071 ± 1.388	1, 17	-0.771	0.451

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