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1 Short telomeres drive pessimistic judgment bias in 2 zebrafish

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10 Abstract

11 The role of telomerase reverse transcriptase (TERT) has been widely investigated in the
12 contexts of aging and age-related diseases. Interestingly, decreased telomerase activities
13 (and accelerated telomere shortening) have also been reported in patients with emotion-
14 related disorders, opening the possibility for subjective appraisal of stressful stimuli
15 playing a key role in stress-driven telomere shortening. In fact, patients showing a
16 pessimistic judgment bias have shorter telomeres. However, these evidences in humans
17 are correlational and the causal directionality between pessimism and telomere shortening
18 of telomeres has not been established experimentally yet. We have developed and
19 validated a judgment bias experimental paradigm to measure subjective evaluations of
20 ambiguous stimuli in zebrafish. This behavioural assay allows classifying individuals in
21 an optimistic-pessimistic dimension (i.e. from individuals that consistently evaluate
22 ambiguous stimuli as negative to others that perceived them as positive). Using this
23 behavioural paradigm we found that telomerase-deficient zebrafish (*tert*^{-/-}) were more

24 pessimistic in response to ambiguous stimuli than WT zebrafish. The fact that individuals
25 with constitutive shorter telomeres have pessimistic behaviours demonstrate for the first
26 time in a vertebrate model a genetic basis of judgment bias.

27 **Keywords:** judgment bias, pessimistic, telomere shortening, telomerase reverse
28 transcriptase (TERT), zebrafish

29

For Review Only

30 **Introduction**

31 Telomeres are complexes of repetitive DNA sequences and proteins that together act as
32 caps at chromosome ends protecting them from deterioration. Telomere shortening during
33 mitosis is prevented by telomerase, an enzyme that adds 6-bp DNA repeat sequences
34 [(TTAGGG) n] to telomeres. In humans, telomerase expression is restricted in somatic
35 cells so that telomeres shorten during lifespan [1]. Telomere shortening is also accelerated
36 by chronic psychological stress and may serve as an indicator for stress-related disease
37 susceptibility (e.g. [2]). The subjective appraisal of stressful stimuli has been suggested
38 to play a key role in stress-driven telomere shortening, since pessimistic individuals have
39 shorter telomeres [3]. However, the available evidence in humans is correlational and the
40 causal inference of pessimism driving shortening of telomeres has not been established
41 experimentally yet. On the other hand, decreased telomerase activities (and accelerated
42 telomere shortening) have been also reported in patients with emotion-related disorders
43 [4, 5], which opens the possibility for a bidirectional link between pessimism and
44 telomere attrition.

45 Judgment biases of ambiguous stimuli also occur in animals, with some individuals
46 consistently evaluating them as negative (aka pessimists) and others as positive (aka
47 optimists) [6]. Judgment bias has been conceptualized as a decision-making process that
48 is modulated by the affective state of the individual, such that a negative emotional state
49 is predicted to induce a pessimistic assessment of ambiguous stimuli [7, 8]. Therefore,
50 judgment bias has been seen mainly as a phenotypic state influenced by the current
51 affective state of the individual that depends to a great extent to the environment in which
52 the individual is exposed, rather than a constitutive phenotypic trait, while research on
53 the genetic component of judgment bias is scarce (e.g. [9, 10]). In this respect, the

54 availability of mutant lines for the telomerase reverse transcriptase (TERT) [11], which
55 is the core catalytic protein component of telomerase, opens the possibility of testing the
56 hypothesis that pessimism may be causally driven by telomere attrition (i.e. TERT
57 mutants with shorter telomeres have constitutive pessimistic bias). In fact, a recent study
58 in mice has reported that specific valenced-emotions, such as depressive-like states, are
59 deeply influenced by the action of TERT [11]. Considering the emotional modulation of
60 judgment biases, these intriguing findings on TERT open the possibility for telomerase-
61 deficient individuals exhibiting also alterations in the judgment biases that produce
62 subjective evaluations. We have tested this hypothesis by using a *tert*^{-/-} mutant in zebrafish
63 (*Danio rerio*), which offers an ideal model to unravel this question since: (1) its stress
64 axis is well characterised and is highly conserved as compared to that of mammals [12];
65 (2) pharmacological validation of standard behavioural tests of anxiety-like behaviour
66 have been reported in this species [13];(3) it is a short-lived fish species with human-like
67 telomere sizes that, like in humans, requires telomerase for a normal lifespan and tissue
68 homeostasis [14].

69 **Material and methods**

70 ***Fish and Housing***

71 Two independent experiments were carried out in this study: Experiment 1 aimed to
72 validate a go/no-go judgement bias task in zebrafish, and Experiment 2 assessed the effect
73 of telomerase deficiency on judgement bias. Fish used for Experiment 1 were 4 months-
74 old male wild-type (Tübingen strain) zebrafish (*Danio rerio*) (n = 14 fish). In Experiment
75 2, age-matched individuals of the telomerase mutant line *tert*^{AB/hu3430} (see *Supplementary*
76 *material* for further details of this mutant line) and of the WT line were used to assess the
77 role of telomerase in the modulation of judgment biases at two different ages [4 or 9

78 months-old; n = 10-12 male fish per Genotype (WT or *tert*^{-/-}) and Age]. A between-
79 individuals design with respect to age was used (i.e. fish from different breeding sources
80 were tested at 4 and 9 month-old). All fish were bred and held at Instituto Gulbenkian de
81 Ciência (IGC, Oeiras, Portugal) (see *Supplementary material* for more details on housing
82 procedures). After behavioural testing, fish were kept in their housing tank to be reused
83 in future experiments since procedures described here did not cause significant
84 impairment of the wellbeing or general condition of the animals. All procedures were
85 performed in accordance with Institutional and National regulations and guidelines,
86 reviewed by the Ethics Committee of the Instituto Gulbenkian de Ciência, and approved
87 by the competent Portuguese authority (Direcção Geral de Alimentação e Veterinária).

88 ***Experimental procedures***

89 ***Experiment 1: Validation of the judgment bias test*** – In this study, we have developed
90 and validated a judgment bias test for zebrafish, which has been designed as a Go/No-go
91 task, based on the judgment bias experimental paradigm published by Harding et al. [6].
92 The behavioural apparatus consisted of a half arm radial maze (Fig. 1A) with guillotine
93 doors linking the starting box with each arm. The two reference arms (P and N) were
94 positioned 180° from each other. Coloured cards (green or red) were associated with each
95 of these arms. The three ambiguous arms (NP, A and NN) were positioned at equidistant
96 angles between the two reference arms and associated with mixed coloured cards (colour
97 proportions of 3:1, 1:1 and 1:3, respectively). After a habituation period to the apparatus,
98 fish were trained in the two reference arms to perform one response [Positive (P)] when
99 one cue was presented (specific location/colour cue) in order to experience a positive
100 event (food reward; see *Supplementary material* for further details). Furthermore, fish
101 were also trained to perform a different response [Negative (N)] when presented with a
102 different cue in order to avoid a negative event (chasing with net; see *Supplementary*

103 *material* for further details). For technical reasons, the responses used in this study were
104 lower (P) or higher (N) latencies to enter the experimental arms of the behavioural
105 apparatus. Once fish were able to discriminate between P and N arms (as indicated by
106 different latencies to enter each one), their responses to ambiguous arms between P and
107 N (NP, A, and NN) were tested (i.e. ambiguous probe trials). This type of experimental
108 paradigm has been used in a wide range of species to assess judgement bias [e.g. 15-22]
109 (see *Supplementary material* for the detailed protocol of judgment bias assay for
110 zebrafish).

111 ***Experiment 2: Effect of telomerase deficiency in judgement bias***– The experimental
112 procedure validated in Experiment 1 was used in experiment 2 to assess the effect of
113 telomerase deficiency on judgement bias. Since an accurate discrimination performance
114 between stimuli (presumably by a generalization response) was demonstrated in
115 Experiment 1, a shorter Test phase omitting NP and NN cue testing was implemented in
116 Experiment 2 (see *Supplemental information* for further details). A shorter Test phase
117 and, consequently, a lower number of training trials in this phase could have a number of
118 advantages in terms of minimizing potential events affecting the categorization of the
119 ambiguous cue. For instance, a higher number of positive outcomes (i.e. food rewards)
120 may lead to a decrease in appetite, which could affect the performance of optimistic
121 behaviours independently of the affective state. Appetite impact on judgment bias tasks
122 has been already reported [23, 24]. On the other hand, a higher confounding influence of
123 stress could be achieved by increasing the number of negative outcomes (i.e.
124 punishments) and/or the overall duration of the Test phase. The effects of stress on task
125 learning in judgment bias tests have also been previously reported [25, 26].

126 ***Behavioural observations***

127 The latency to enter in the target arm (60 s maximum) was recorded for each trial. Video
128 recordings were analyzed using a multi-event recorder software (The Observer XT,
129 Noldus technology, version 9). Behavioural data were analyzed by one researcher and
130 scored blindly to Genotype, Age and Treatment.

131 *Statistical analyses*

132 For the analyses of both experiments – validation of the judgment bias paradigm
133 (Experiment 1) and the judgment bias test in the *tert*^{-/-} mutants (Experiment 2) – we used
134 the R software [27] packages “lme4” [28] and “afex” [29] for the linear mixed effects
135 models (GLMM), and the “emmeans” package [30] for planned comparisons. Details of
136 R codes used and the original datasets can be found online [31]. The response variables
137 were the latencies to respond to stimuli, that is, the time it took the fish to enter the
138 experimental arms [positive (P), near positive (NP), ambiguous (A), near negative (NN),
139 negative (N) in experiment 1 and P, A and N in experiment 2] of the behavioural
140 apparatus. Latencies were restricted to the interval between 0 and 60 seconds and were
141 log-transformed. In the model of Experiment 1, the fixed effect was Treatment, with five
142 groups (P, NP, A, NN, N). In the model of Experiment 2, the fixed effects were Treatment
143 (with three groups: P, N, A) in interaction with Age (with two groups: 4 and 9 months of
144 age) and Genotype (with two groups: wild type and *tert*^{-/-}). In both models, the random
145 effect was the individual fish, since the same individuals were tested in all treatments
146 within each experiment and for each age group (but different sets of individuals were used
147 for the two age groups). Inspection of model residuals from both experiments showed
148 satisfactory normal distributions. All P-values are two-tailed except when indicated
149 otherwise (i.e. when an *a priori* directional hypothesis is provided).

150 **Results**

151 ***Experiment 1: Validation of the judgment bias test in zebrafish***

152 WT zebrafish showed significant differences in the latency for each Treatment (P, NP, A,
153 NN, N) (Fig. 1B; GLMM: $F_{4,52} = 27.626$, $p < 0.001$), reflecting a generalization response
154 with fish showing a lower latency to enter the P arm and progressively increasing the
155 latency as the colour cue/location neared N. This stimuli generalization suggests that
156 individuals are categorizing the different cues as predicting the associated event to each
157 one, hence displaying appropriate responses.

158 ***Experiment 2: Effect of telomerase deficiency in judgement bias***

159 *Tert*^{-/-} zebrafish mutants were tested for judgment bias and compared with age matched
160 WT fish for two different ages (4 or 9 months-old; Fig. 1C). Statistical analysis of
161 Experiment 2 showed that only Treatment had a significant main effect (Table 1),
162 reflecting the generalization response described in Experiment 1. There was also an
163 interaction effect between Genotype and Age (Table 1), with *tert*^{-/-} mutants displaying a
164 more pessimistic bias than wild-type at younger age, and such differences disappearing
165 in older fish (Fig. 1C). There was also an interaction between Age and Treatment (Table
166 1), with older WT showing more pessimistic bias (Fig. 1C). It is important to note that
167 both WT and *tert*^{-/-} mutants showed similar latencies to enter the P and N reference arms
168 (Fig. 1C), indicating that the observed differences in the response towards the A arm
169 result from judgment biases rather than from altered sensorimotor abilities that may also
170 be compromised in *tert*^{-/-} mutants.

171 Since other judgement bias studies have used a Judgment Bias Score in their analyses
172 (e.g. [7, 32]) we also ran such an analysis in parallel, which yielded similar results (see
173 *Supplementary material* for further details; Fig. 1SA). Based on the judgement bias score
174 animals can be classified into optimistic and pessimistic. In line with the GLMM analysis

175 presented above, the proportion of pessimistic individuals was significantly higher in
176 *tert*^{-/-} mutants than in WT fish at 4 months old (0.60, n=10 vs. 0.25, n=12; z=-1.66, one-
177 tailed p= 0.049, Fig. 1SB), and in WT at 9 months than in WT at 4 month old (0.818,
178 n=11 vs. 0.25, n=12; z=-2.72, one-tailed p<0.01; Fig. 1SB).

179 **Discussion**

180 In this study, we have measured optimism/pessimism for individual zebrafish by using a
181 judgment bias task that has been designed to measure expectations of positive (reward)
182 and negative (punishment) outcomes when fish are exposed to ambiguous stimuli
183 intermediate between two stimuli previously associated with reward and punishment.
184 Before discussing the results obtained from the judgment bias paradigm, a number of
185 issues related to our behavioural task need to be considered. Cues commonly used in
186 judgment bias paradigms are spatial (e.g., [7]), visual (e.g., [19]), olfactory (e.g., [17]), or
187 auditory (e.g., [20]). However, we have used a combination of different stimulus classes
188 (i.e. spatial and visual), which has been successfully operated in bumblebees [15]. The
189 use of more than one class of stimulus may facilitate the acquisition of the available
190 information related to the task, and hence improve discrimination learning. This fact
191 could be critical to overcome the difficulties showed by zebrafish in categorizing more
192 than one stimulus simultaneously and, consequently, in successfully performing
193 judgment bias paradigms [33]. On the other hand, our results suggest the occurrence of a
194 basic psychological mechanism namely stimulus generalization, which has been proven
195 to play an important role in responses to ambiguous stimuli in judgment bias paradigms
196 (e.g. [15-19]). The occurrence of this mechanism is indicative of an accurate
197 discrimination between a stimulus (or set of stimuli) that predicts a positive consequence
198 and a stimulus (or set of stimuli) that predicts a negative one.

199 The majority of the studies conducted to date on judgment bias have focused on the effects
200 of manipulations that are expected to induce a negative affective state (e.g., [34, 35]). In
201 fact, Baciadonna and McElligott [36] suggest that judgment bias tasks are highly sensitive
202 to manipulations that produce negative emotions. Here we show that at 4 months of age
203 zebrafish *tert*^{-/-} mutants display more pessimistic-like behaviours in response to
204 ambiguous cues as compared with WT zebrafish. Considering the above-mentioned
205 studies, it can be hypothesized that the pessimistic-like judgment bias displayed by
206 telomerase-deficient zebrafish could be indicative of a negative affective state associated
207 with *tert* silencing. *tert*^{hu3430} homozygous mutant strain in zebrafish (*tert*^{-/-}) has been
208 shown to have shorter telomeres than WT siblings as consequence of the absence of
209 telomerase [14]. These mutant zebrafish develop degenerative phenotypes from 4 – 6
210 months onwards and die prematurely. The development of such early phenotypic
211 alterations (e.g. increased inflammation), which are common in aged organisms, may be
212 responsible for the altered judgment bias performances of the telomerase-deficient
213 mutants. In fact, a correlational link between pessimism and inflammation has already
214 been reported in humans [37]. Similarly, the increased pessimism in older WT fish is also
215 paralleled by an aging related shortening of telomeres that WT fish experience. In fact,
216 Henriques et al. [14] also found that telomeres decrease in length over time during the
217 first year of life of *tert*^{+/+} zebrafish, which is accompanied by deterioration of physical
218 state. However, at 9 months of age *tert*^{-/-} mutants and WT fish still differ in their telomere
219 lengths, and hence the most plausible explanation for the lack of a difference in pessimism
220 at older age is the existence of a threshold in terms of tissue homeostasis, above which
221 the judgement bias phenotype is similarly affected. Together these results suggest that a
222 decrease in physical state and/or in life span signalled by telomerase is associated with a
223 pessimistic judgement bias. This result is in line with the current theories that link life-

224 history strategy to affective states in animals and humans, according to which the adaptive
225 function of mood (and their dependent judgment bias) is to integrate information about
226 the recent state of the environment and of physical condition of the organism in order to
227 optimize behavioural decision-making [38]. Accordingly, individuals that are physically
228 compromised are less able to cope with undetected threats if they should arise, and should
229 behave more cautiously towards ambiguous stimuli [38]. Similarly, individuals with short
230 life spans are expected to follow a fast life history strategy, which has been associated
231 with depression syndromes in humans [39, 40]. Together, this evidence supports our
232 initial hypothesis, that *tert* mutants with shorter telomeres have constitutive pessimistic
233 bias, which may be interpreted in the scope of adaptive life history theory.

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240 **Data Accessibility**

241 The dataset used in this study is available at the public data repository Dryad at
242 <https://doi.org/10.5061/dryad.rv15dv46m>.

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362

363 **Figure Captions**

364 **Figure 1.** Judgment bias in zebrafish. **(A)** Diagram of the experimental setup showing the
365 two reference locations (i.e. positive/rewarded (P) and negative/aversive (N)) and the
366 three ambiguous locations (i.e. near positive (NP), ambiguous (A) and near negative
367 (NN)). Each location is associated to a specific colour cue. The test consists in training
368 the fish to discriminate between the P and the N location/colour cue. Once fish are able
369 to discriminate between them (as indicated by different latencies in entering in each one),
370 their responses to ambiguous locations/colour cues between the positive and the negative
371 are tested. **(B)** Mean latencies during the test phase (Experiment 1) on trials performed
372 for the P and N training locations/colour cues, and for the three ambiguous
373 locations/colour cues (NP, A, and NN; n = 14 male fish); Different letters indicate
374 significant differences between groups following post-hoc multiple comparisons tests.
375 **(C)** Performance of *tert*^{-/-} mutants and WT siblings (Experiment 2) at different ages [n =
376 10-12 male fish per Genotype (WT or *tert*^{-/-}) and Age (4 or 9 months-old)] in the
377 judgment bias paradigm. Different letters indicate significant differences between

378 genotype and age groups for each Treatment (P, A, N) following planned comparisons
 379 tests. Data are expressed as mean \pm SEM.

380

381 **Tables**

382 Table 1. Results of the General Linear Mixed Model to assess the effects of Treatment
 383 (positive vs. negative vs. ambiguous), Genotype (wild-type vs. *tert* mutant), Age (4
 384 months old vs. 9 months old), and the double and triple interactions among these
 385 variables; * indicates a significant effect.

Main effects and interactions	F value	P (> F)
Genotype	$F_{1,40} = 2.14$	$p = 0.15$
Age	$F_{1,40} = 3.22$	$p = 0.08$
Treatment	$F_{2,80} = 169.04$	$p < 0.001$ *
Genotype x Age	$F_{1,40} = 4.35$	$p < 0.05$ *
Genotype x Treatment	$F_{2,80} = 1.48$	$p = 0.23$
Age x Treatment	$F_{2,80} = 7.63$	$p < 0.001$ *
Genotype x Age x Treatment	$F_{2,80} = 2.10$	$p = 0.13$

386

387 **Supplementary figure caption**

388 **Figure S1.** Judgment bias in zebrafish. **(A)** Judgment Bias Score (JBS) and **(B)**
 389 percentage of optimistic and pessimistic of *tert*^{-/-} mutants and WT siblings (Experiment
 390 2) at different ages (n = 10-12 male fish per Genotype (WT or *tert*^{-/-}) and Age (4 or 9

391 months-old)). A higher JBS indicates a response to the ambiguous stimulus that is more
392 similar to the response to the positive stimulus than to the negative one. JBS data are
393 expressed as mean \pm SEM.

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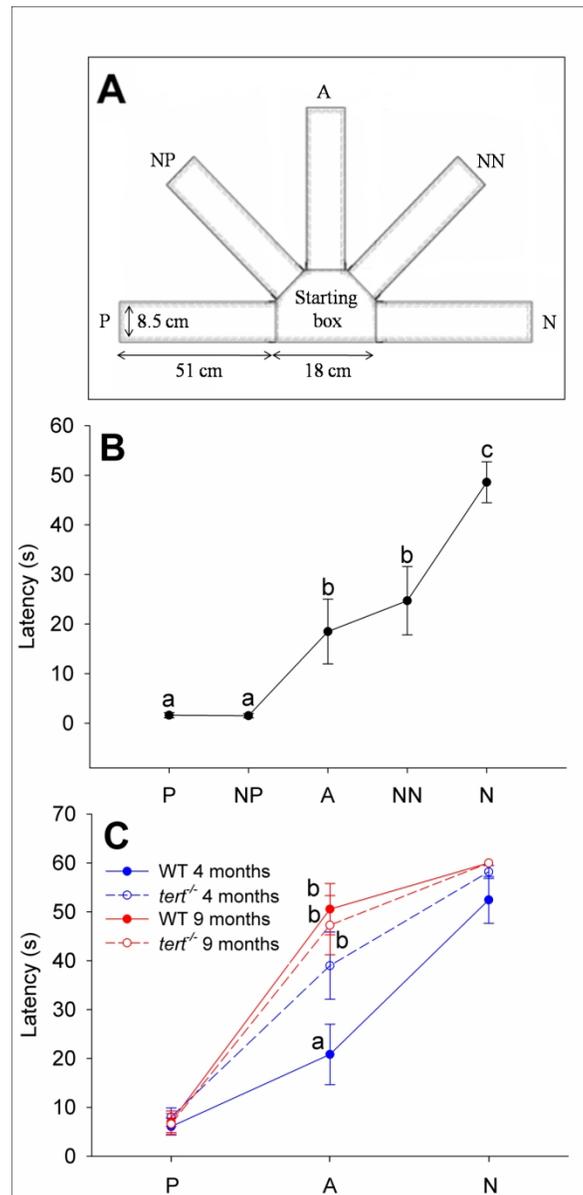


Fig 1

191x390mm (150 x 150 DPI)

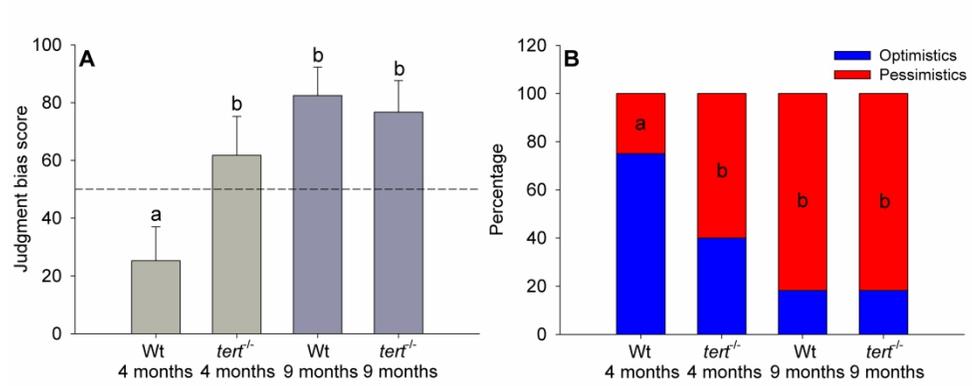


Fig S1

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