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Short telomeres drive pessimistic judgment bias in zebrafish

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Abstract

The role of telomerase reverse transcriptase (TERT) has been widely investigated in the contexts of aging and age-related diseases. Interestingly, decreased telomerase activities (and accelerated telomere shortening) have also been reported in patients with emotion-related disorders, opening the possibility for subjective appraisal of stressful stimuli playing a key role in stress-driven telomere shortening. In fact, patients showing a pessimistic judgment bias have shorter telomeres. However, these evidences in humans are correlational and the causal directionality between pessimism and telomere shortening of telomeres has not been established experimentally yet. We have developed and validated a judgment bias experimental paradigm to measure subjective evaluations of ambiguous stimuli in zebrafish. This behavioural assay allows classifying individuals in an optimistic-pessimistic dimension (i.e. from individuals that consistently evaluate ambiguous stimuli as negative to others that perceived them as positive). Using this behavioural paradigm we found that telomerase-deficient zebrafish (tert⁻/⁻) were more
pessimistic in response to ambiguous stimuli than WT zebrafish. The fact that individuals with constitutive shorter telomeres have pessimistic behaviours demonstrate for the first time in a vertebrate model a genetic basis of judgment bias.

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

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

Judgment biases of ambiguous stimuli also occur in animals, with some individuals consistently evaluating them as negative (aka pessimists) and others as positive (aka optimists) [6]. Judgment bias has been conceptualized as a decision-making process that is modulated by the affective state of the individual, such that a negative emotional state is predicted to induce a pessimistic assessment of ambiguous stimuli [7, 8]. Therefore, judgment bias has been seen mainly as a phenotypic state influenced by the current affective state of the individual that depends to a great extent to the environment in which the individual is exposed, rather than a constitutive phenotypic trait, while research on the genetic component of judgment bias is scarce (e.g. [9, 10]). In this respect, the
availability of mutant lines for the telomerase reverse transcriptase (TERT) [11], which
is the core catalytic protein component of telomerase, opens the possibility of testing the
hypothesis that pessimism may be causally driven by telomere attrition (i.e. TERT
mutants with shorter telomeres have constitutive pessimistic bias). In fact, a recent study
in mice has reported that specific valenced-emotions, such as depressive-like states, are
deeply influenced by the action of TERT [11]. Considering the emotional modulation of
judgment biases, these intriguing findings on TERT open the possibility for telomerase-
deficient individuals exhibiting also alterations in the judgment biases that produce
subjective evaluations. We have tested this hypothesis by using a tert\(^{-/-}\) mutant in zebrafish
(Danio rerio), which offers an ideal model to unravel this question since: (1) its stress
axis is well characterised and is highly conserved as compared to that of mammals [12];
(2) pharmacological validation of standard behavioural tests of anxiety-like behaviour
have been reported in this species [13];(3) it is a short-lived fish species with human-like
telomere sizes that, like in humans, requires telomerase for a normal lifespan and tissue
homeostasis [14].

Material and methods

Fish and Housing

Two independent experiments were carried out in this study: Experiment 1 aimed to
validate a go/no-go judgement bias task in zebrafish, and Experiment 2 assessed the effect
of telomerase deficiency on judgement bias. Fish used for Experiment 1 were 4 months-
old male wild-type (Tübingen strain) zebrafish (Danio rerio) (n = 14 fish). In Experiment
2, age-matched individuals of the telomerase mutant line tert\(^{AB/nu3430}\) (see Supplementary
material for further details of this mutant line) and of the WT line were used to assess the
role of telomerase in the modulation of judgment biases at two different ages [4 or 9
months-old; n = 10-12 male fish per Genotype (WT or tert−/−) and Age]. A between-individuals design with respect to age was used (i.e. fish from different breeding sources were tested at 4 and 9 month-old). All fish were bred and held at Instituto Gulbenkian de Ciência (IGC, Oeiras, Portugal) (see Supplementary material for more details on housing procedures). After behavioural testing, fish were kept in their housing tank to be reused in future experiments since procedures described here did not cause significant impairment of the wellbeing or general condition of the animals. All procedures were performed in accordance with Institutional and National regulations and guidelines, reviewed by the Ethics Committee of the Instituto Gulbenkian de Ciência, and approved by the competent Portuguese authority (Direcção Geral de Alimentação e Veterinária).

**Experimental procedures**

**Experiment 1: Validation of the judgment bias test** – In this study, we have developed and validated a judgment bias test for zebrafish, which has been designed as a Go/No-go task, based on the judgment bias experimental paradigm published by Harding et al. [6]. The behavioural apparatus consisted of a half arm radial maze (Fig. 1A) with guillotine doors linking the starting box with each arm. The two reference arms (P and N) were positioned 180° from each other. Coloured cards (green or red) were associated with each of these arms. The three ambiguous arms (NP, A and NN) were positioned at equidistant angles between the two reference arms and associated with mixed coloured cards (colour proportions of 3:1, 1:1 and 1:3, respectively). After a habituation period to the apparatus, fish were trained in the two reference arms to perform one response [Positive (P)] when one cue was presented (specific location/colour cue) in order to experience a positive event (food reward; see Supplementary material for further details). Furthermore, fish were also trained to perform a different response [Negative (N)] when presented with a different cue in order to avoid a negative event (chasing with net; see Supplementary
material for further details). For technical reasons, the responses used in this study were lower (P) or higher (N) latencies to enter the experimental arms of the behavioural apparatus. Once fish were able to discriminate between P and N arms (as indicated by different latencies to enter each one), their responses to ambiguous arms between P and N (NP, A, and NN) were tested (i.e. ambiguous probe trials). This type of experimental paradigm has been used in a wide range of species to assess judgement bias [e.g. 15-22] (see Supplementary material for the detailed protocol of judgment bias assay for zebrafish).

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

**Behavioural observations**

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

Statistical analyses

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

Results

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Experiment 1: Validation of the judgment bias test in zebrafish

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

Experiment 2: Effect of telomerase deficiency in judgement bias

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

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

$tert^{-/-}$ mutants than in WT fish at 4 months old (0.60, n=10 vs. 0.25, n=12; $z=-1.66$, one-
tailed $p=0.049$, Fig. 1SB), and in WT at 9 months than in WT at 4 month old (0.818,
$n=11$ vs. 0.25, n=12; $z=-2.72$, one-tailed $p<0.01$; Fig. 1SB).

**Discussion**

In this study, we have measured optimism/pessimism for individual zebrafish by using a
judgment bias task that has been designed to measure expectations of positive (reward)
and negative (punishment) outcomes when fish are exposed to ambiguous stimuli
intermediate between two stimuli previously associated with reward and punishment.
Before discussing the results obtained from the judgment bias paradigm, a number of
issues related to our behavioural task need to be considered. Cues commonly used in
judgment bias paradigms are spatial (e.g., [7]), visual (e.g., [19]), olfactory (e.g., [17]), or
auditory (e.g., [20]). However, we have used a combination of different stimulus classes
(i.e. spatial and visual), which has been successfully operated in bumblebees [15]. The
use of more than one class of stimulus may facilitate the acquisition of the available
information related to the task, and hence improve discrimination learning. This fact
could be critical to overcome the difficulties showed by zebrafish in categorizing more
than one stimulus simultaneously and, consequently, in successfully performing
judgment bias paradigms [33]. On the other hand, our results suggest the occurrence of a
basic psychological mechanism namely stimulus generalization, which has been proven
to play an important role in responses to ambiguous stimuli in judgment bias paradigms
(e.g. [15-19]). The occurrence of this mechanism is indicative of an accurate
discrimination between a stimulus (or set of stimuli) that predicts a positive consequence
and a stimulus (or set of stimuli) that predicts a negative one.
The majority of the studies conducted to date on judgment bias have focused on the effects of manipulations that are expected to induce a negative affective state (e.g., [34, 35]). In fact, Baciadonna and McElligott [36] suggest that judgment bias tasks are highly sensitive to manipulations that produce negative emotions. Here we show that at 4 months of age zebrafish tert−/− mutants display more pessimistic-like behaviours in response to ambiguous cues as compared with WT zebrafish. Considering the above-mentioned studies, it can be hypothesized that the pessimistic-like judgment bias displayed by telomerase-deficient zebrafish could be indicative of a negative affective state associated with tert silencing. tert^{h3430} homozygous mutant strain in zebrafish (tert−/) has been shown to have shorter telomeres than WT siblings as consequence of the absence of telomerase [14]. These mutant zebrafish develop degenerative phenotypes from 4 – 6 months onwards and die prematurely. The development of such early phenotypic alterations (e.g. increased inflammation), which are common in aged organisms, may be responsible for the altered judgment bias performances of the telomerase-deficient mutants. In fact, a correlational link between pessimism and inflammation has already been reported in humans [37]. Similarly, the increased pessimism in older WT fish is also paralleled by an aging related shortening of telomeres that WT fish experience. In fact, Henriques et al. [14] also found that telomeres decrease in length over time during the first year of life of tert^{+/+} zebrafish, which is accompanied by deterioration of physical state. However, at 9 months of age tert−/− mutants and WT fish still differ in their telomere lengths, and hence the most plausible explanation for the lack of a difference in pessimism at older age is the existence of a threshold in terms of tissue homeostasis, above which the judgement bias phenotype is similarly affected. Together these results suggest that a decrease in physical state and/or in life span signalled by telomerase is associated with a pessimistic judgement bias. This result is in line with the current theories that link life-
history strategy to affective states in animals and humans, according to which the adaptive
function of mood (and their dependent judgment bias) is to integrate information about
the recent state of the environment and of physical condition of the organism in order to
optimize behavioural decision-making [38]. Accordingly, individuals that are physically
compromised are less able to cope with undetected threats if they should arise, and should
behave more cautiously towards ambiguous stimuli [38]. Similarly, individuals with short
life spans are expected to follow a fast life history strategy, which has been associated
with depression syndromes in humans [39, 40]. Together, this evidence supports our
initial hypothesis, that tert mutants with shorter telomeres have constitutive pessimistic
bias, which may be interpreted in the scope of adaptive life history theory.

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Data Accessibility

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

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**Figure Captions**

**Figure 1.** Judgment bias in zebrafish. (A) Diagram of the experimental setup showing the two reference locations (i.e. positive/rewarded (P) and negative/aversive (N)) and the three ambiguous locations (i.e. near positive (NP), ambiguous (A) and near negative (NN)). Each location is associated to a specific colour cue. The test consists in training the fish to discriminate between the P and the N location/colour cue. Once fish are able to discriminate between them (as indicated by different latencies in entering in each one), their responses to ambiguous locations/colour cues between the positive and the negative are tested. (B) Mean latencies during the test phase (Experiment 1) on trials performed for the P and N training locations/colour cues, and for the three ambiguous locations/colour cues (NP, A, and NN; n = 14 male fish); Different letters indicate significant differences between groups following post-hoc multiple comparisons tests. (C) Performance of tert<sup>−/−</sup> mutants and WT siblings (Experiment 2) at different ages [n = 10-12 male fish per Genotype (WT or tert<sup>−/−</sup>) and Age (4 or 9 months-old)] in the judgment bias paradigm. Different letters indicate significant differences between
genotype and age groups for each Treatment (P, A, N) following planned comparisons tests. Data are expressed as mean ± SEM.

Tables

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

| Main effects and interactions | F value   | P (> F)   |
|-------------------------------|----------|-----------|
| Genotype                     | F1,40= 2.14 | p = 0.15  |
| Age                          | F1,40= 3.22 | p = 0.08  |
| Treatment                    | F2,80= 169.04 | p < 0.001 * |
| Genotype x Age               | F1,40= 4.35 | p < 0.05 * |
| Genotype x Treatment         | F2,80= 1.48 | p = 0.23  |
| Age x Treatment              | F2,80= 7.63 | p < 0.001 * |
| Genotype x Age x Treatment   | F2,80= 2.10 | p = 0.13  |

Supplementary figure caption

Figure S1. Judgment bias in zebrafish. (A) Judgment Bias Score (JBS) and (B) percentage of optimistic and pessimistic of tert−/− mutants and WT siblings (Experiment 2) at different ages (n = 10-12 male fish per Genotype (WT or tert−/−) and Age (4 or 9
months-old)). A higher JBS indicates a response to the ambiguous stimulus that is more similar to the response to the positive stimulus than to the negative one. JBS data are expressed as mean ± SEM.
Fig 1

191x390mm (150 x 150 DPI)
Fig S1

308x125mm (300 x 300 DPI)