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Intraspecific variation in freshwater tolerance has consequences for telomere dynamics in the euryhaline teleost *Dicentrarchus labrax*

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Abstract :

Stressful events can alter organism physiology at several levels triggering allostatic responses. Telomeres are well-conserved repetitive DNA sequences mainly localised at chromosome's ends, playing a crucial role in DNA stability. Analyses of telomere dynamics are new tools to assess consequences of environmental stress in non-model organisms like fish. In this study, the relationship between freshwater tolerance and telomere dynamics was investigated in the gills of the European sea bass Dicentrarchus labrax. Fluorescent in situ hybridisation of telomeric sequences revealed distal telomeres as well as intrachromosomal telomeres known as interstitial telomere sequences. In order to better understand telomere dynamics in the gills of D. labrax, we used quantitative PCR to measure telomere length and mRNA expression of the catalytic subunit of telomerase reverse transcriptase tert. For the calculation of the relative telomere length, two reference genes were tested: the single copy gene mc2r, encoding melanocortin 2 receptor and the multicopy gene 18S, encoding the 18S ribosomal RNA. We proposed a novel normalisation method to calculate the relative telomere length using both, single and multiple copy genes as references. Cell dynamics was also investigated by measuring mRNA expression of genes involved in apoptosis (caspase 8 and 9), cell proliferation (proliferation cell nuclear antigen), aerobic mitochondrial metabolism (ATP citrate-synthase), anaerobic metabolism (lactate dehydrogenase a) and antioxidant enzymatic defences (superoxide dismutase 1 and 2, catalase). Following a 15-days fresh water exposure, telomere dynamics was not significantly modified in the gills of freshwater tolerant fish. But freshwater intolerant fish exhibited telomere attrition relative to saltwater controls, and lower expression of tert in gills relative to freshwater tolerant fish. This modification of telomere dynamics in intolerant individuals was found to be correlated with lower antioxidant enzymatic defences, a higher aerobic metabolic marker and a lower cellular turnover. These data bring new perspectives for the use of telomere dynamics as an integrative marker to study environmental stress in fish, while considering individual phenotypic plasticity in response to freshwater exposure.

Highlights

► Freshwater exposure triggered telomere attrition in freshwater intolerant *D. labrax* ► Freshwater intolerant showed a lower telomerase mRNA expression than tolerant fish ► Telomere dynamics was linked with cell dynamics, metabolism and antioxidant markers

Keywords : Telomere length, Telomerase reverse transcriptase mRNA expression, Freshwater tolerance, Cell dynamics, Energy metabolism, Oxidative stress

Introduction

Marine organisms living in fluctuating environments such as lagoons and estuaries have to constantly deal with abiotic stressors (salinity, temperature, oxygen). A strong physiological plasticity is required to be able to face salinity, temperature and oxygen level fluctuations (Claireaux and Lagardère 1999). The European sea bass *Dicentrarchus labrax* (Linnaeus, 1758) is a demersal fish of high commercial interest which inhabits coastal waters. *D. labrax* enters estuaries, lagoons and sometimes ascending rivers most likely to feed (Rogdakis et al. 2010). In *D. labrax*, a strong intra-specific variability was highlighted regarding its capacity to tolerate hyperthermia (Ozolina et al. 2016), hypoxia (Claireaux et al., 2013; Joyce et al., 2016) and freshwater exposure (Nebel et al., 2005, L'Honoré et al., 2019, 2020), suggesting an inter-individual difference in the capacity to tolerate harsh environmental conditions. Burton and Metcalfe (2014) highlighted that exposure to stressful conditions in early life stages can have long-term and inter-generational effects on physiology and fitness in several taxa, including fish. It is questionable whether repeated stress encountered throughout life by fish such as *D. labrax* migrating seasonally in transitional waters, has a negative impact on fitness.

Few studies have examined the potential use of telomere length as an integrative marker of stress exposure in fish (Anchelin et al. 2013; Henriques et al. 2013; Naslund et al. 2015; Debes et al. 2016). Telomeres are well conserved terminal regions of eukaryotic chromosomes, composed of repetitive sequences of TTAGGG in vertebrates (Blackburn and Gall 1978). Telomeres ensure multiple functions in preserving chromosome stability, including protecting the ends of chromosomes from degradation and preventing chromosomal end fusion (Blackburn 1991). Telomere length (TL) and telomerase activity are commonly used to study ageing in higher vertebrates (Aubert and Lansdorp 2008; Saretzki 2018). Telomerase plays a crucial role in chromosome stability and cell viability by extending the

distal 3' end of eukaryotic linear chromosome over replications (Blackburn 2005). This enzymatic complex consists of the telomerase reverse transcriptase (TERT) catalytic subunit, the telomerase RNA component (TERC) involved in the replication of the telomere sequence, and other associated proteins contributing to elongate telomeres localised at the end of the chromosomes (Blackburn 2005; Smith et al. 2020). In most non-mammal species such as birds and fish, telomere dynamics relies on two opposite forces: telomere attrition and telomere restoration, supported by telomerase. In human, chronic oxidative stress and life stressors can accelerate telomere attrition by decreasing telomerase activity or *tert* expression levels (Epel et al. 2004; Houben et al. 2008; Starkweather et al. 2014). In ecological studies, telomere length provides a mechanistic link between environmental condition, life history traits and fitness (Monaghan and Haussmann 2006; Haussmann 2010; Monaghan 2014; Mathur et al. 2016). According to recent meta-analyses focused on ecological studies in non-model vertebrates (Angelier et al. 2018; McLennan et al. 2018; Wilbourn et al. 2018), we still lack crucial basic data to fully understand: (i) the influence of abiotic factors, such as salinity or temperature, on telomere length, (ii) the intra-specific variation in telomere dynamics and the drivers of this intra-specific variation and (iii) the potential link between telomere attrition, lifespan and mortality risk, especially in bony fish species.

The effect of temperature on telomere attrition was the main environmental abiotic parameter analysed in fish. In mosquitofish *Gambusia holbrooki*, a decrease from 25°C to 20°C for 24 h was associated with a decrease in telomere length (Rollings et al. 2014). Conversely, an increase in temperature from 20°C to 30°C for 1 month triggered telomere attrition in the Siberian sturgeon *Acipenser baerii* (Simide et al. 2016). Regarding the relationship between telomere attrition and ageing in fish, studies are controversial. In the zebrafish, telomere length has been observed to increase from larvae to adult stages and to shorten significantly in older individuals (Anchelin et al. 2013). Additionally, Hatakeyama et al. (2008) showed in the

95 medaka that telomeres do not shorten linearly with age, but shortening dynamics depends on
96 growth rate and level of telomerase activity at each life stage. Therefore, it appears that
97 telomere dynamics is particularly variable and nonlinear in fish.

98 Previous experimental studies performed in juvenile *D. labrax* at different ages have shown
99 that about 25 to 30% of individuals are unable to acclimate successfully to experimental
100 transfer from seawater to fresh water (Nebel et al. 2005; L'Honoré et al. 2019, 2020). The
101 freshwater intolerant phenotype exhibits several characteristics: failure in hydromineral
102 balance regulation, decrease in swimming capacities, downregulation of gluco- and mineralo-
103 corticoid receptors involved in both stress response and osmoregulation and, ultimately, death
104 (Nebel et al., 2005, L'Honoré et al., 2019, 2020). Recently, Angelier et al. (2018) raised new
105 hypotheses suggesting a trade-off between immediate survival and telomere
106 maintenance/protection, which would transitionally lead to shortened telomeres during an
107 "emergency state". In this study, we compare extreme phenotypes regarding freshwater
108 tolerance (tolerant *vs* intolerant) in order to determine if *D. labrax* exhibiting contrasted
109 freshwater tolerance differ in telomere dynamics.

110 The gill was considered as a somatic tissue of interest to study the relationship between hypo-
111 osmotic stress and telomere dynamics as the branchial epithelium exhibits a rapid cell
112 turnover and a strong morphological plasticity (Nilsson 2007; Kang et al. 2013). In *D. labrax* ,
113 gills are able to remodel within 1 to 2 weeks in response to fluctuations of environmental
114 factors like salinity, oxygen availability and temperature (Sollid and Nilsson 2006; Lorin-
115 Nebel et al. 2006; Nilsson et al. 2012; Masroor et al. 2018). Such plasticity in the response to
116 environmental change has been demonstrated to be associated to elevated cellular dynamics,
117 such as cell renewal and apoptosis (Sollid 2005; Tzaneva et al. 2014; Sales et al. 2017;
118 Mierzwa et al. 2020). In addition, an increased number of gill mitochondrion-rich cells
119 (MRCs) has been shown in hypo-osmotic environments in numerous species including *D.*

120 *labrax* (Nebel et al. 2005; Masroor et al. 2018), suggesting a raise of energetic demand to fuel
121 active ion transport (Evans et al. 2005). Interestingly, freshwater intolerant *D. labrax* were
122 previously characterised by a higher density of branchial MRCs compared to freshwater
123 tolerant fish (Nebel et al. 2005), suggesting metabolic disorders in freshwater intolerant *D.*
124 *labrax* .

125 Since mitochondria are known to be the main source of ROS production in cells (Lambert and
126 Brand 2009), an increase in mitochondria may also trigger an increased production of
127 metabolic ROS, as a by-product of cellular respiration (Quijano et al. 2016). *In vitro,*
128 oxidative stress was shown to be a major factor triggering DNA damage and accelerated
129 telomere shortening in human endothelial cells, through the reduction of telomerase activity
130 (Kurz et al. 2004; Ahmed and Lingner 2017). *In vivo* studies showing a direct link between
131 oxidative stress and telomere dynamics are more scarce (Boonekamp et al. 2017). Recent
132 reviews by Reichert and Stier (2017) and Chatelain et al. (2020) concluded that there is strong
133 evidence from both experimental and correlative *in vivo* studies in vertebrates that oxidative
134 stress induces effects on telomere dynamics, with tissue-dependent, life stage-dependant and
135 sex-dependant variations. Nevertheless, more experimental studies are required to further
136 understand the influence of oxidative stress on telomere dynamics *in vivo*.

137 The first aim of this study was to determine the occurrence and the localisation of telomeres
138 in *D. labrax* genome using fluorescence *in situ* hybridisation (FISH) in order to test if
139 interstitial telomeric sites are detected. The head kidney was used for karyotyping because of
140 its high cell renewal (Bertollo et al. 2015). Then, an acute 2 weeks freshwater stress was used
141 to test whether osmotic stress affects telomere dynamics in the gills of 5-month-old *D. labrax*
142 exhibiting contrasted freshwater tolerance capacities, as previously described in L'Honoré *et*
143 *al.* (2019). Telomere attrition was evaluated using relative TL measurement using q-PCR and
144 the mRNA expression of *tert* was measured as a proxy of telomere maintenance. To better

understand cell dynamics and the potential influence of oxidative stress and energy metabolism on telomere dynamics, mRNA expression of genes involved in apoptosis (*caspase 8* and *9*), cell proliferation (*proliferation cell nuclear antigen*), aerobic mitochondrial metabolism (*ATP citrate-synthase*), anaerobic metabolism (*lactate dehydrogenase a*) and antioxidant enzymatic defences (*superoxide dismutase 1* and *2*, *catalase*) were measured. Osmotic stress and individual tolerance to fresh water may differentially influence telomere dynamics where telomere attrition would reflect the harshness of the environment an individual has experienced. We hypothesised that non-tolerant fish to fresh water will exhibit shorter telomeres than tolerant fish, as a consequence of oxidative and physiological stress. If telomeres shorten to critical levels in the gill tissue, this may trigger organ dysfunction, as previously shown in gut and muscle zebrafish (Carneiro et al. 2016). This could have consequence on general physiology and survival since the fish gill is a multifunctional organ involved in gas exchange, osmoregulation, acid-base regulation, and excretion of nitrogenous waste (Evans et al. 2005). As short telomeres induce senescence in cells and hence reduce the regenerative capacity of the corresponding tissues, it has been suggested that TL might affect various fitness parameters (Monaghan and Haussmann 2006). In fact, TL has been linked to survival and reproductive success in some bird species (Haussmann et al. 2005; Pauliny et al. 2006). From an evolutionary ecology point of view, telomere-induced selection could occur if telomere attrition differently affects relative fitness among individuals (Olsson et al. 2017). Evidence for causal effects of telomere traits on life history and fitness-related parameters is still limited. In this study, we investigate the consequence of an acute osmotic stress in the non-model euryhaline species *D. labrax* to test whether intraspecific differences in osmoregulatory capacities have consequences on TL maintenance.

Materials and methods

1. Origin of animals

Fish were issued from *in vitro* fertilisation of unrelated wild native West Mediterranean breeders (40 males and 23 females) in order to obtain a large genetic diversity. Sea bass were grown at the Ifremer Station at Palavas-les-flots (Hérault, France) under a 16/8 hours light/dark photoperiod in seawater (SW) at 20°C. Food was proposed *ad libidum.*

2. Fluorescence *in situ* hybridisation on telomere sequence DNA and microscope analysis

Karyotype analysis and fluorescent *in situ* hybridisation were performed at the CytoEvol facilities of UMR ISEM of the LabEx CeMEB (Montpellier, France). Cephalic kidney of two males and 2 females (10 month-old) were sampled and processed as described in Ozouf-Costaz *et al.* (2015). Fluorescence *in situ* hybridisation (FISH) was performed following the same procedure as described in Ozouf-Costaz *et al.* (2015), using an oligonucleotide telomeric probe $(TTAGGG)_7$ labelled with Cy3 at its 5' end (biomers.net, Ulm, Germany) and counterstaining the chromosomes with DAPI (4',6-diamidino-2-phenylindole)-antifade mounting medium solution (Vectashield, Vector Laboratories, Peterborough, UK). Three slides were prepared per individual and preparations were analysed using a Zeiss Axioplan 2 Imaging epifluorescence microscope equipped with a cooled charge couple devise camera and Cytovision 7.4 software (Applied Imaging, San Jose, CA).

3. Experimental exposure to freshwater

Five month-old *D. labrax* juveniles (N=1525, 4.20 ± 0.09 cm, 0.87 ± 0.06 g) were experimentally exposed to fresh water according to L'Honoré *et al.* (2019). Briefly, fish were transferred from SW to brackish water (BW) at 15 ppt for 24h before being transferred to fresh water (FW) for 2 weeks. A no replication experimental setup, where intolerant fish and tolerant fish are maintained in the same tank and exact same conditions, was chosen because

we expected from previous studies that the FW intolerant phenotype represents about one third of the experimental cohort (Nebel et al. 2005; L'Honoré et al. 2019, 2020), thus requiring an elevated number of animals (N=1525). In addition, the detection of FW intolerant phenotype also requires an elevated number of individuals swimming in shoals in order to be able to observe abnormal individual behaviour within the shoal as described in L'Honoré et al. (2019). After 2 weeks of freshwater challenge, tolerant and intolerant phenotypes were sorted, measured and weighted. More precisely, fish exhibiting erratic swimming, isolation from the shoal associated with low reflexes and stronger pigmentation were identified as the freshwater-intolerant phenotype (FW-I). These animals were characterised by an incapacity to maintain hydromineral balance in FW (Nebel et al., 2005; L'Honoré et al. 2019, 2020). The three experimental groups analysed were: seawater controls (SW, 6.30 ± 0.12 cm, 2.84 ± 0.14 g), freshwater tolerant fish (FW-T, 5.28 ± 0.10 cm, 1.47 ± 0.10 g) and freshwater intolerant fish (FW-I, 5.20 ± 0.10 cm, 1.25 ± 0.08 g). At the end of the exposure, fish were euthanised in 100 ppm of benzocaine and the first left gill arc was dissected, flash frozen in liquid nitrogen and stored respectively dry or in RNA*later* (Quiagen, Valencia, CA) at -80°C until gDNA and mRNA extraction.

4. gDNA extraction

Genomic DNA (gDNA) extraction was performed using the Maxwell® 16 Buccal Swab LEV DNA Purification Kit (Promega, Charbonières, France). Samples were eluted in 50 µL of ultrapure water. Quantity was measured fluorometrically using a Qubit dsDNA BR Assay Kit (Thermo Fisher Scientific), concentrations ranged from 60 to 200 µg mL^{-1}. Purity was verified using the NanoDrop™ One/One^{C} Spectrophotometer (Thermo Scientific, Waltham, MA, USA) through A260/A280 and A260/A230 ratios. DNA quality was checked using Bioanalyzer 2100 (Santa Clara, CA, United States).

5. RNA extraction and reverse transcription

RNA extraction was performed using the total RNA extraction kit that includes a DNase step (Nucleospin® RNA, Macherey-Nagel, Germany). Quantity and purity of extraction products were verified using a UV spectrophotometer (NanoDrop™ One/OneC Spectrophotometer, Thermo Scientific, Waltham, MA, USA). RNA quality was checked using Bioanalyzer 2100 and RIN levels were comprised between 6 and 9 (mean RIN = 8.15). Reverse transcription was performed using one microgram of RNA using the qScript™ cDNA SuperMix (Quanta Biosciences™) providing all necessary components for first-strand synthesis: buffer, oligo(dT) primers, random primers and qScript reverse transcriptase.

6. Target genes selection

Key genes were selected to better understand cell dynamics and the potential influence of oxidative stress and energy metabolism on telomere dynamics. Proliferating cell nuclear antigen (*pcna*) was used as a cell proliferation marker (Sadoul et al. 2018) whereas caspase 8 and 9 (*casp 8*, *casp 9*) were used as extrinsic and intrinsic cell apoptosis markers respectively (Olsson and Zhivotovsky 2011; Paiola et al. 2018). Mitochondrial superoxide dismutase 1 (*sod1*), cytosolic superoxide dismutase 2 (*sod2*) and catalase (*cat*) were selected as antioxidant enzymes because superoxide anion ($O_2^{\cdot -}$) and hydrogen peroxide (H_2O_2) are the main ROS formed by mitochondria. Lactate dehydrogenase is a key enzyme in the control of energy metabolism composed of four polypeptide subunits encoded by two genes: *ldh-a* and *ldh-b* (Driedzic et al. 1980). In this study, *ldh-a* was investigated as a marker for anaerobic glycolysis (Almeida-Val et al. 2011; Valvona et al. 2016). Gene encoding citrate synthase (*cs*) was selected as a marker of aerobic metabolism (Roche and Reed 1974; Elcock and McCammon 1996; Goldenthal et al. 1998).

7. Quantitative real-time polymerase chain reaction

Telomere length measurement and relative mRNA gene expression quantification was realised using 384-wells plates filled with an Echo®525 liquid handling system (Labcyte Inc., San Jose, CA, USA). Each well contained a mix composed by 1.5 µL of LightCycler-FastStart DNA Master SYBR-Green I™ Mix (Roche, Manheim, Germany), 0.27 µL of each primer (forward and reverse primers at 0.9 µM final concentration), 0.23 µL of ultrapure water and 1 µL of cDNA or gDNA. For *pcna, tert, casp8, casp9, sod1, sod2, cat, cs, ldh-a, 18S* and *l13* cDNA amplification, efficiency (E) of each primer pair was tested using standard curves performed on all-samples pools of cDNA (Table 1).

For mRNA expression analyses, the q-PCR conditions were as follows: 2 min denaturation at 95 °C followed by 35 cycles (95 °C for 30 s, 61 °C for 45 s and 72 °C for 1 min) followed by a final elongation step at 72 °C for 4 min. The reference genes *18S* and *l13* were chosen according to previous studies performed in sea bass (Mitter et al. 2009). Relative mRNA expressions were normalised against two reference genes, *l13* and *18S,* according to the method of Vandesompele et al. (2002) and expressed using the comparative ΔΔCt method (Ct, threshold cycle number) described by Pfaffl (2001), with SW fish as a reference. For all samples, measurements were run in triplicates, and no-template control (water) Ct was above 40.

For TL measurement, the q-PCR conditions were adapted from Cawthon (2009) with some modifications as follows: 15 min denaturation at 95 °C followed by 2 cycles (94° C for 15 s, 49° C for 15 s) followed by 35 cycles (95 °C for 15 s, 62 °C for 10 s and 74 °C for 15 sec). Telomere primers used to amplify telomeric hexamer repeats were TEL G and TEL C as described in Cawthon (2009). Efficiency of each primer pairs reported on Table 1 were obtained by standard curves performed on all-sample pools of gDNA (*tel*, *mc2r* and *18S*). Relative TL calculation was performed using the ratio between telomere repeat copy number and reference gene copy number known as T/R ratio. T/R ratio was calculated with the ΔΔCt

method described in Cawthon (2002) and normalised against two reference genes, a single copy gene *mc2r* and a multicopy gene *18S* (Wang et al. 2013).

Formulas used "E" as primers efficiency as indicated in Table 1. The condition SW was used as the control condition for the ΔΔCt calculation. An inter-plate assay was performed to investigate the potential variability between two different q-PCR runs. Inter-assay validation was performed in duplicates with *mc2r* and *18S* on gDNA of 16 samples.

8. Statistics

Statistical analyses were performed on GraphPad Prism (version 6, GraphPad Software Incorporated, La Jolla, CA 268, USA). First, Grubb's test was used on the 15 fish per condition to remove the potential outliers from the data set. Since data fitted normality test (D'Agostino-Pearson test) but not homoscedasticity test (Bartlett test), Mann-Whitney pairwise comparisons were performed, with Bonferroni adjustment ($p < 0.0167$). For inter-plate assay correlation analysis, Pearson correlation tests were used because data fitted with normality assumption. A non-parametric Spearman correlation test was performed to study the correlation between quantitative variables. Experimental values are reported as means ± s.e.m..

Results

1. FISH of DNA telomere sequences in *D. labrax* karyotypes

Karyotype analyses confirmed the presence of 2n=48 chromosomes (Fig. 1) as expected in *D. labrax* (Sola et al., 1993). Fluorescent in situ hybridisation of telomeric sites revealed that telomere sequences were localised distally, as expected. Interstitial telomeric sequences (ITS) localised proximally were also observed. FISH does not allow a precise detection so we

cannot conclude about any inter-individual differences in signal intensity or localisation of telomere sequences.

2. Relative telomere length measurements

2.1 Method validation

Primer efficiency of the single copy gene *mc2r* and the multicopy gene *18S* were at 2.0 (Table 1). The primers specificity was checked using the melting point (T_m) of the product for each primer pair and displayed a unique pike at the expected temperature. The inter-plate Pearson correlation r^2 were respectively above 0.92 and 0.99 for the T/R ratio with *mc2r* and *18S* as reference genes (Pearson test, $P < 0.0001$ for each gene, Figs 1Sa-b). Coefficients of variation (CV) did not exhibited values > 3% for both intra-assay CV and inter-assay CV as resumed in Table 1S.

Regarding TL, CV were the highest using *18S* as the reference gene (32.0% in SW, 34.7% in FW-T and 60.0% in FW-I, Fig. 1Sc), whereas they were the lowest using *mc2r* as the reference gene (26.6% in SW, 35.5% in FW-T and 32.0% in FW-I, Figure 2). Thus, we will consider *mc2r* as the best reference gene for TL since it was 3-times less variable within phenotypes.

2.2 Telomere dynamics in response to freshwater exposure: telomere length and mRNA expression of *tert*

No significant difference in TL could be measured between SW and FW-T ($P = 0.8107$, Fig. 2). A significantly lower relative TL was measured in FW-I compared to FW-T and SW ($P < 0.0001$, Fig. 2). Regarding *tert* expression, no significant difference was measured between SW and FW-T ($P = 0.2115$, Fig. 3a). In FW-I, *tert* expression was significantly lower than in FW-T but not compared to SW ($P = 0.0011$ and $P = 0.072$ respectively).

2.3 mRNA expression of genes involved in cell dynamics, metabolism and antioxidant defences

Transcript levels of *pcna* did not differ between SW and FW-T (P = 0.9144), whereas they were significantly lower in FW-I than in SW and in FW-T (P = 0.0001 and P = 0.0003, Fig. 3b). Although we did not measure any significant difference in *casp8* expression levels between SW and FW-T (P = 0.1936, Fig. 3c), we measured significant lower expression of *casp8* in FW-I compared to FW-T but not compared to SW (P = 0.0137 and P = 0.0367 respectively). We did not measure any significant difference in *casp9* expression levels between the three groups (Fig. 4d).

Superoxide dismutase *sod1* and *sod2* mRNA gene expression did not exhibit any significant differences between the three phenotypes (Figs 4a-b). Regarding *cat*, no significant differences were inferred between SW and FW-T (P = 0.0455). However, FW-I displayed significantly lower expression compared to both SW and FW-T (P = 0.0005 and P = 0.0052 respectively, Fig. 4c).

Concerning *cs* mRNA expression levels, they were significantly lower in SW than in FW-T and FW-I (P = 0.0133 and P = 0.0101, Fig. 4d). However, no significant differences could be inferred between FW-T and FW-I (P = 0.6932), or between each group regarding *ldh-a* mRNA expression levels (P = 0.0469 for SW *vs* FW-T, P = 0.0219 for SW *vs* FW-I, and P = 0.3669 for FW-T vs FW-I).

3. Correlation between variables

Testing the Spearman coefficient correlation between all quantitative variables (Table 2), it appeared that telomere dynamics markers (TL and *tert* mRNA expression) were significantly and positively correlated (r = 0.48, P = 0.013). Body mass and body length were not correlated with telomere dynamics markers ($P > 0.05$ for both), and no significant differences

could be inferred between the two phenotypes in FW (Mann-Whitney test, P = 0.3110 and unpaired t-test, P = 0.5893, for body mass and body length respectively). These correlations were all positives regarding cellular turnover markers (r = 0.48 between *pcna* and TL, r = 0.38 between *casp8* and TL, r = 0.64 between *casp8* and *tert*, r = 0.47 between *casp9* and *tert*) and antioxidant enzymatic defences (r = 0.66 between *sod2* and *tert*, r = 0.59 between TL and *cat*). However, the correlation between TL and metabolic marker was negative (r = -0.58 between *cs* and TL, r = -0.39 between *ldh-a* and TL).

Discussion

1. Method validation

As reviewed in Lai *et al.* (2018), the estimation of relative TL using the q-PCR method may be biased by inter-assays variations. By reproducing the same q-PCR amplification using two different plates as described in Appleby (2016), we showed that the operational variability was very limited in this study. Karyotype analysis of *D. labrax* revealed 24 pairs of chromosomes different in size as already demonstrated in the literature (Sola et al. 1993). In this study, we demonstrate the presence of interstitial telomeric sequences (ITS) in *D. labrax*. According to Ocalewicz (2013), most of the pericentromeric and ITS in fish are possible relicts of chromosome fusion events. The occurrence of ITS may potentially reduce the sensitivity of the q-PCR method for TL measurement by adding a background noise, especially if small TL changes are expected. Due to their intrachromosomal position, several authors suggested that ITS do not shorten during DNA replication or in response to ageing or stress (Foote et al. 2013). According to a recent meta-analysis of Chatelain et al. (2020), the noise in telomere length resulting from interstitial repeats may not mask the differences in the length of end-cap telomeres between individuals, using the qPCR method or the TRF method. It would be interesting to use a quantitative technique such as Q-FISH (Lai et al. 2018) to

360 further explore the proportion of ITS *vs* terminal telomeric sequences and to determine
361 whether the inter-individual variability of ITS is elevated in sea bass.

362 For relative quantification, the choice of the reference gene may be crucial to improve the
363 reliability of the T/R ratio calculation. While most studies used single copy genes as reference
364 for relative TL calculation (Lai et al., 2018), Wang et al. (2013) demonstrated in single cells
365 that a multicopy gene like *18S* was more robust for this calculation. However, given that the
366 variability among each group seemed to depend on the reference gene, we propose to use a
367 single copy gene like *mc2r* to reduce bias due to the variations of a single specific reference
368 gene.

369 2. Telomere and cell dynamics in gills following FW exposure

370 The use of TL as a biomarker for environmental stress exposure requires a tissue with active
371 telomere dynamics. Previous study working on erythrocytes reported no difference in
372 telomere length in *D. labrax* with age (Horn et al. 2008). Gill tissue has several interesting
373 properties: a strong plasticity associated with high cell renewal and active cell division
374 (Nilsson 2007; Tzaneva et al. 2014), the presence of MRCs suggesting an active cellular
375 respiration and potential increased production of metabolic ROS by-products (Hwang and Lee
376 2007). To our knowledge, the effect of salinity change on telomere dynamics has never been
377 studied in euryhaline teleost. In this study, we were able to detect a significant TL reduction
378 of about 50% in the gill of FW-I after only two weeks of freshwater stress. A strong inter-
379 individual variability in TL was observed, as expected in vertebrates (Dugdale and
380 Richardson 2018; Toupance et al. 2019). This highlights that telomere dynamics in gill is
381 quickly modified. In accordance to the hypothesis of Angelier et al. (2018), TL was not
382 maintained in fish whose survival is threatened, suggesting a trade-off between immediate
383 survival and telomere protection.

Interestingly, the quick telomere attrition measured in FW-intolerant fish was correlated to a significant lower *tert* expression compared to FW-tolerant fish, suggesting an altered capacity to maintain TL in intolerant fish facing freshwater stress. Conversely, transfer from seawater to fresh water did not trigger any significant change in *tert* expression and TL in FW_T. In European hake *Merluccius merluccius* and in Atlantic cod *Gadus morhua*, *tert* expression was found higher in early developmental stages suggesting an higher telomerase demand possibly linked with elevated tissue renewal and long-term cell proliferation capacity maintenance (López de Abechuco et al. 2014). However, there is no clear trend concerning the relationship between ageing and telomerase activity (Hatakeyama et al. 2008; Henriques et al. 2013; Saretzki 2018). Regarding cell dynamics, freshwater transfer is expected to increase cell population renewal associated with branchial epithelium remodelling occurring during hypo-osmotic acclimation (Nilsson 2007; Masroor et al. 2018). We observed no significant changes in mRNA expression of cell dynamics markers of apoptosis *casp8, casp9* or proliferation *pcna* in FW-T after 2 weeks of exposure. Most of the cellular changes have probably been completed in successfully acclimated *D. labrax* within 2 weeks of freshwater exposure (Nebel et al. 2005). In the gill of FW-I, *casp8* and *pcna* levels were significantly down-regulated compared to the freshwater tolerant condition, suggesting a slowdown of cell dynamics in the gills of intolerant fish. This is consistent with the results of Carneiro et al. (2016), which observed a decreased cell proliferation in the gut and testis of tert-/- mutants zebrafish using PCNA immunostaining. Conversely, in cellular *in vitro* models, a link between *tert* overexpression, cell survival and increased cell proliferation has been shown (Dagarag et al. 2004; Aubert and Lansdorp 2008). Given that we highlighted a correlation between cellular dynamics and telomere dynamics in *D. labrax*, we can hypothesise that the reduction of cellular dynamics observed in FW-I may be associated to a reduction of *tert* expression or telomerase activity. The reduction of cell dynamics in intolerant fish may be possibly due to

409 an exhaustion of energetic reserves allocated to osmoregulatory processes. Due to technical
410 and ethical limitations regarding the number of individuals used for this study, no replication
411 of freshwater and seawater treatment was performed. Therefore, we cannot exclude a batch
412 effect between SW and FW fish or other confounding factor that may influence within salinity
413 treatment response.

414 3. Metabolism and antioxidant defences following freshwater exposure

415 Freshwater exposure differentially affected energy metabolism and antioxidant enzymatic
416 defences in *D. labrax* according to their individual freshwater tolerance capacity. Freshwater
417 exposure significantly increased mRNA gene expression of the citrate synthase gene, a
418 marker of aerobic metabolism. Acclimation of teleosts to different environmental salinities
419 causes depletion of energy which is used to regulate the functioning of various highly energy-
420 consuming pumps and ion transporters in gill MRCs (Chang et al. 2007; Hwang and Lee
421 2007; Tseng and Hwang 2008). In tilapia gill epithelial cells, Tseng et al. (2008) have shown
422 that citrate synthase and LDH proteins were induced after transfer from FW to SW,
423 confirming the active role of these enzymes to fuel active ion-pumping and fish
424 osmoregulation. During salinity challenges either from SW to FW or from FW to SW, an
425 increase in lactate contents and LDH activities has been reported in the gills of several
426 euryhaline teleost fish (Vijayan et al. 1996; Polakof et al. 2006; Tseng et al. 2008) indicating
427 the involvement of monocarboxylate metabolites in gill energy consumption during
428 osmoregulation. In this study, no significant changes in mRNA expression of *ldh-a* could be
429 inferred according to the Bonferonni adjusted *p*-value of 0.0169. Present mRNA gene
430 expression data should be taken with caution since they do not reflect the concentration and/or
431 activity of the related protein. Therefore, additional biochemical analyses (e.g. activity of key
432 enzymes of aerobic and anaerobic metabolic pathways such as LDH, citrate synthase or
433 citrate oxidase) would be necessary to confirm the hypothesis of a metabolic distress in FW-

intolerant fish. But data from this and previous studies (Nebel et al. 2005; L'Honoré et al. 2019, 2020) converge to this hypothesis. After two weeks in FW, the cost of acclimation is maintained elevated in FW-I compared to FW-T and SW. This is consistent with results of previous studies in sea bass showing that (i) intolerant fish over-absorbed ions in the gills to compensate a renal failure (L'Honoré et al., 2020), (ii) intolerant fish exhibit and overabundance of MRCs in gills (Nebel et al. 2005) and (iii) intolerant fish exhibit a change in gluco- and mineralocorticoids regulatory pathways, underlying impairment of hydro-mineral balance and stress response regulation (L'Honoré et al., 2020). Thus, in species exhibiting intraspecific variability in abiotic stress tolerance such as salinity in killifish *Fundulus heteroclitus* (Scott and Schulte 2005), temperature (Ozolina et al. 2016) or hypoxia (Joyce et al. 2016) in *D. labrax*, differences in gill TL should be further investigated to test whether differential patterns of tolerance to physiological stress have consequence on telomere attrition, and possibly on tissue functioning as suggested by Carneiro et al. (2016).

Mitochondria are widely recognized as a source of ROS in animal cells, where it is assumed that overproduction of ROS may conduct to an overwhelmed antioxidant system and oxidative stress (Quijano et al. 2016). Therefore, an elevated mitochondrial metabolism could increase the production of ROS and would therefore require an activation of anti-oxidant defences to maintain the oxidative balance. In this study, the expression of *cat*, *sod1* or *sod2* genes, encoding enzymes involved in the main mitochondrial anti-oxidant defences, were not significantly modified after 2 weeks in freshwater in the gills of the tolerant fish compared to seawater controls. These results are consistent with Ghanavatinasab et al. (2019), where no significant difference in SOD and CAT were observed in yellowfin seabream *Acanthopagrus sheim* exposed for 2 weeks in 5 ppt water. However, a significant decrease in *cat* expression levels was measured in the gills of FW-intolerant sea bass compared to FW-tolerant and SW. This result suggests that telomere attrition in FW-intolerant fish could be due to an imbalance

between increased ROS production and downregulated antioxidant defences, leading to oxidative damage on telomeres in individuals with lower capacity to induce *tert*. But this hypothesis needs to be further explored by investigating pro-oxidants, other enzymatic and non-enzymatic anti-oxidant defences as well as oxidative damages.

This study suggests that, in case of elevated physiological and metabolic stress, telomere repair is not prioritised and that energetic limitation has direct consequence on telomere maintenance. These results are consistent with the hypothesis of Angelier et al. (2018) suggesting a trade-off between immediate protection and telomere maintenance. Additional evidence concerning energy metabolism, oxidative stress and damage would be necessary to support the preliminary results of this study. Another recent *in vivo* study highlighted that TL and metabolism are more tightly linked than initially thought (Casagrande and Hau, 2019). The results obtained in this study are in agreement with the *metabolic telomere attrition concept* proposed by Casagrande and Hau (2019), that assumes that TL attrition is strongest during times of energy limitation. Oxidative stress may also be at stake but the relationship between ROS production and mitochondrial energy production remains to be further investigated (Salin et al. 2015). In marine teleost, there is no evidence that hyposaline stress triggers oxidative stress as shown in hepatic tissue of *D. labrax* (Sinha et al. 2015) as well as in *A. sheim* gills (Ghanavatinasab et al. 2019). But again, the gill was poorly studied and a transient increase of production of metabolic ROS, as a by-product of cellular respiration cannot by excluded. According to these hypotheses, telomere dynamics can be considered as a major determinant for cell homeostasis.

Finally, our results suggested that, in the wild, freshwater environment requiring active ionic regulation would potentially not represent a stress involving telomere shortening in fish having large salinity tolerance capacity, if salinity variation is considered solely. But in transitional waters, other environmental parameters are at stake. In particular, temperature and

hypoxia have been shown to upregulate TERT expression in testis and liver of medaka and decrease TL in muscle and fin in brown trout, respectively (Yu et al. 2006; Debes et al. 2016). Multi-stress experimental studies would be necessary to further understand the influence of abiotic factors on telomere length, but the results obtained from this study bring interesting information regarding the consequences of exposure to harsh low salinity conditions and the intra-specific variation in telomere dynamics on non-model fish vertebrates, which is of particular interest for ecologists in the context of global change. Therefore, an interesting perspective of this work would be to determine whether marked fluctuations of environmental parameters, such as those encountered in transitional waters, affect TL in the wild, in association with other life-history traits markers such as otolithometry in order to gain further information on age, growth rate and habitat (Darnaude and Hunter 2017; Bouchoucha et al. 2018).

Conclusion

The q-PCR method performed in this study was efficient to detect relative telomere length changes in sea bass exposed to freshwater. Differences in telomere dynamics in the gills was linked with individual phenotypic plasticity related to freshwater tolerance. Lower telomere dynamics (telomere length and *tert* expression) in FW-I was correlated with a higher aerobic metabolism as well as a lower antioxidant defences.

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Ethics

The experiments were conducted according to the guidelines of the European Union (directive 86/609) and of the French law (decree 87/848) regulating animal experimentation. The experimental design has been approved by the French legal requirement concerning welfare of experimental animals (APAFIS permit no. 9045-201701068219555).

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Figure 1 Fluorescent in situ hybridisation (FISH) of metaphase chromosomes isolated from head-kidneys of 10 month-old European sea bass using the telomeric probe (TTAGGG)7 labelled with Cy3 at its 5' end, indicated by red colour. 2 males and 2 females were analysed with N = 3 slides per fish. Scale bar: 10µm

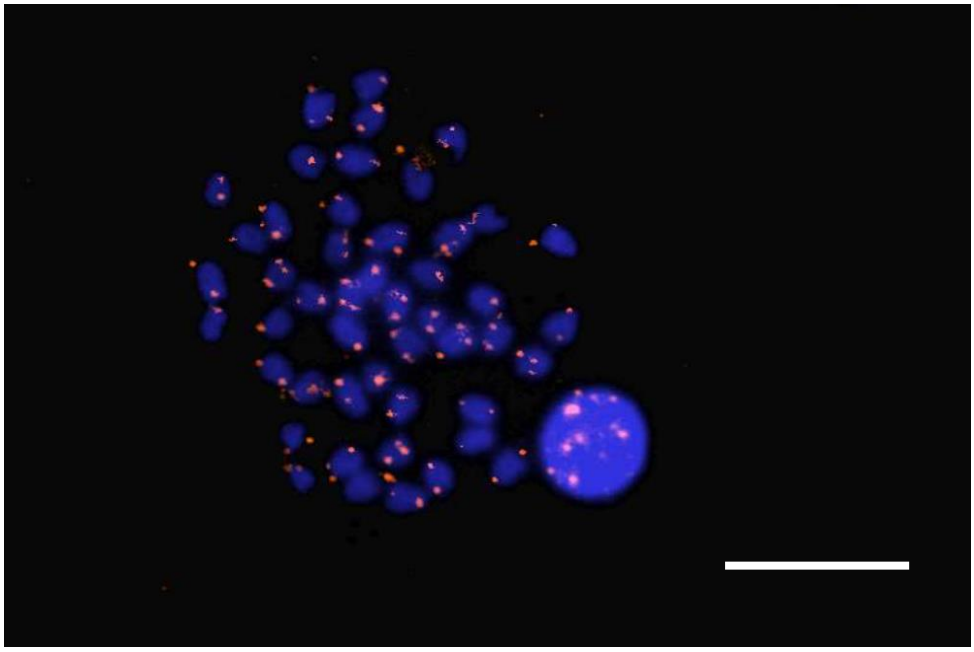

Figure 2 Relative telomere length expressed as T/R ratio calculated using ΔΔCt method normalised against the single copy gene *mc2r* in gills of 5 month-old sea bass maintained in seawater and after a transfer of 2 weeks in fresh water. Different letters denote significant differences between groups (Mann-Whitney test, Bonferroni-corrected $P < 0.0167$, means ± s.e.m, N=10-14). SW: control fish in seawater, FW-T: FW-tolerant fish, FW-I: FW-intolerant fish

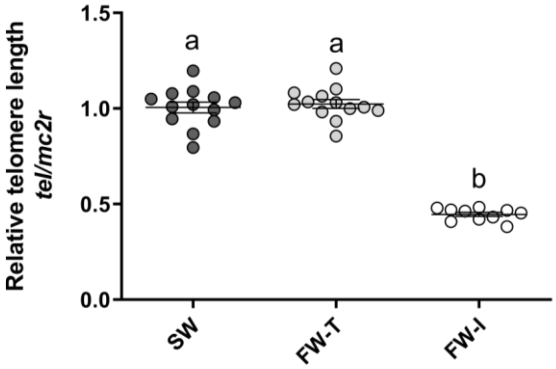

Figure 3 Relative mRNA expression of genes involved in telomere maintenance (a), cell proliferation (b) and apoptosis (c, d) in the gill of 5 month-old sea bass maintained in seawater (SW) or exposed for 2 weeks to fresh water (FW-T and FW-I). (a) telomerase catalytic subunit *tert* (b) proliferation cell nuclear antigen *pcna* (c) caspase 8 *casp8* (d) caspase 9 *casp9*. The mRNA expression was calculated using the ΔΔCt method with SW as a reference and normalised according to the expression of two reference genes *l13* and *18S*. Different letters denote significant differences between phenotypes (Mann-Whitney test, Bonferroni-corrected $P < 0.0167$, means ± s.e.m, N=10-15). SW: control fish in seawater, FW-T: FW-tolerant fish, FW-I: FW-intolerant fish

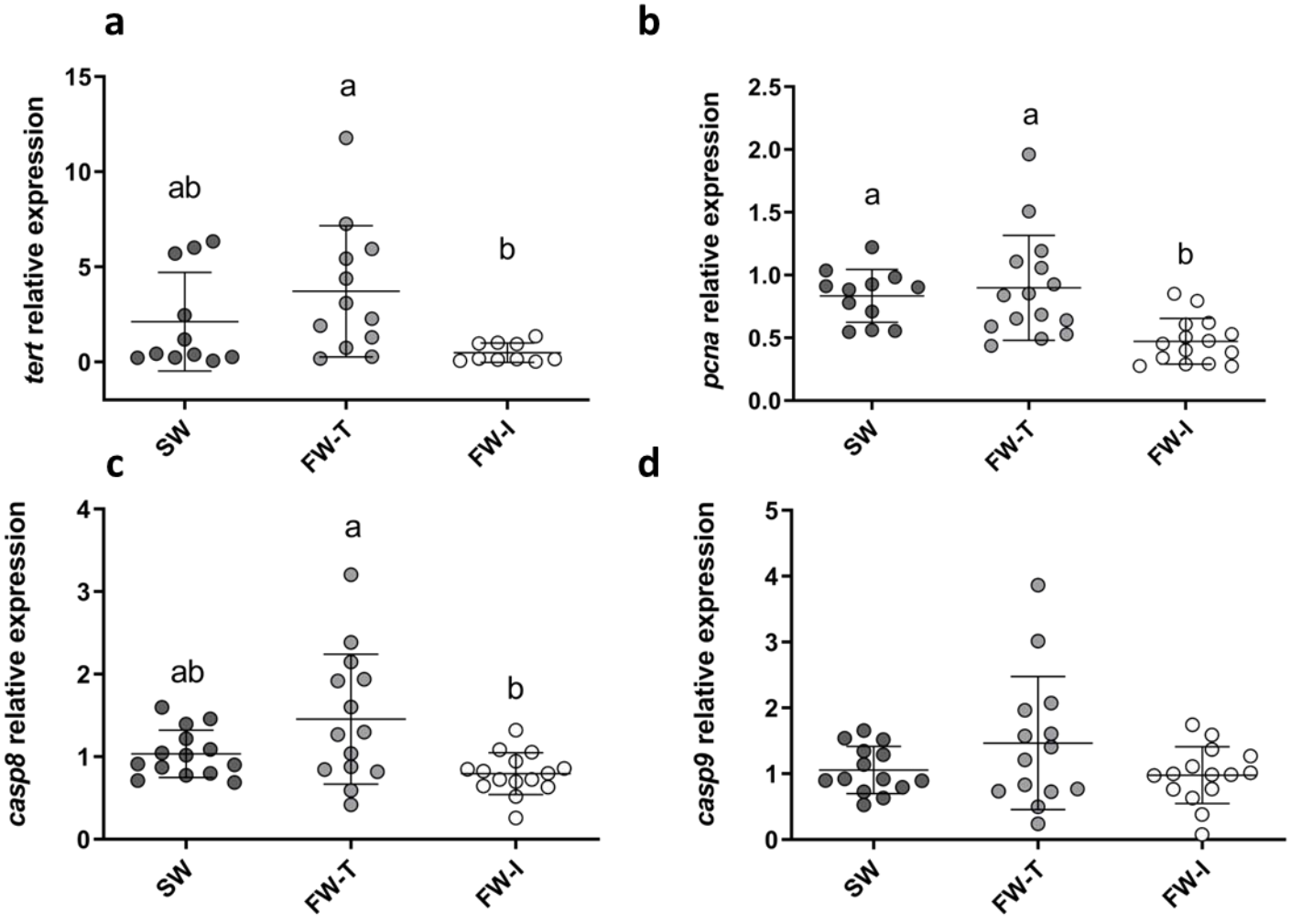

Figure 4 Relative mRNA expression of genes involved in antioxidant defence (a, b, c) and metabolism (d, e) in the gill of 5 month-old sea bass maintained in seawater (SW) or exposed for 2 weeks to fresh water (FW-T and FW-I). (a) superoxide dismutase 1 *sod1* (b) superoxide dismutase 2 *sod2* (c) catalase *cat* (d) ATP citrate synthase *cs* (e) lactate dehydrogenase a *(ldh-a)*. The mRNA expression was calculated using the ΔΔCt method with SW as a reference and normalised according to the expression of two reference genes *l13* and *18S*. Different letters denote significant differences between groups (Mann-Whitney test, Bonferroni-corrected $P < 0.0167$, means ± s.e.m, N=10-15). SW: control fish in seawater, FW-T: FW-tolerant fish, FW-I: FW-intolerant fish

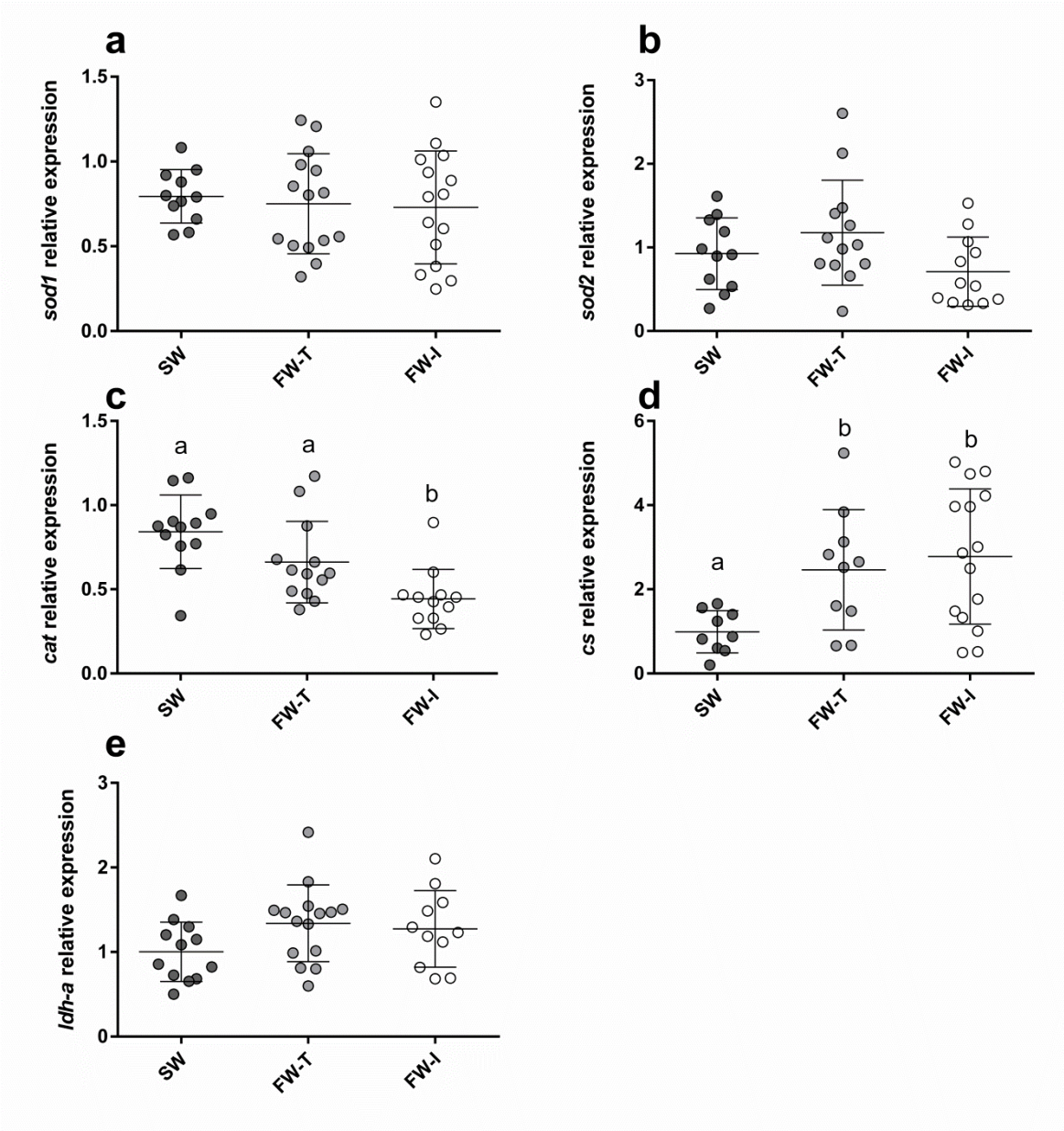

Figure 1S Inter-assay variation of T/R ratio using ΔΔCt method. The two assays were performed separately using 14 genomic DNA samples measured in duplicates. The mean of the duplicate values of each plate was used for T/R ratio calculation. Assay #2 was plotted against Assay #1 in order to study the linear correlation depending on the reference gene used **(a)** *tel / mc2r* (r^2 = 0.9244, Pearson test, $P < 0.0001$) and **(b)** *tel / 18S* (r^2 = 0.9821, Pearson test, $P < 0.0001$). **(c)** Relative telomere length expressed as T/R ratio calculated using ΔΔCt method normalised against the multiple copy gene *18S* in gills of 5 month-old sea bass maintained in seawater and after a transfer of 2 weeks in fresh water. Different letters denote significant differences between groups (Mann-Whitney test, Bonferroni-corrected $P < 0.0167$, means ± s.e.m, N=10-14). SW: control fish in seawater, FW-T: FW-tolerant fish, FW-I: FW-intolerant fish

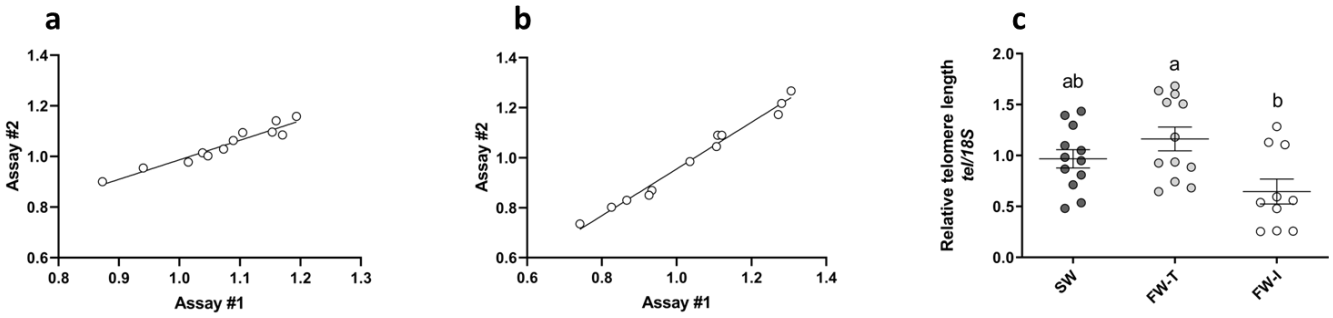

Table 1 Primer sequences used for relative telomere length and gene expression analysis

Target gene	Primer name	Sequences ID	Sequence (from 5' to 3')	Efficiency	Reference
pcna	PCNA F	DLAgn_00120330	CAGAGCGGCTGGTTGCA	1.7	Sadoul et al., 2018
	PCNA R		CACCAAAGTGGAGCGAACAA		
tert	TERT F	DLAgn_00199170	GGGTCAGGGGCTTCTTGTAC	2.1	This study
	TERT R		AGAAACAGGCTCGAACCAGG		
casp8	CASP8 F	FJ225665	TGTCAGGGAAGCCTCTACCA	2.1	Paiola et al., 2018
	CASP8 R		CATCCCCAGCAGGAAGTCAG		
casp9	CASP9 F	DQ345775	CGAATGCAACCGAGCACAAA	1.9	Paiola et al., 2018
	CASP9 R		ACTAACGACCGCCAATGAGG		
tel	TEL G		ACACTAAGGTTTGGGTTTGGGTTTGGGT TTGGGTTAGTGT	2	Cawthon et al., 2009
	TEL C		TGTTAGGTATCCCTATCCCTATCCCTAT CCCTATCCCTAACA	2	
l13	L13 F	DT044539	TCTGGAGGACTGTCAGGGGCATGC	2	Mitter et al., 2009
	L13 R		AGACGCACAATCTTGAGAGCAG		
mc2r	MC2R F	FR870225	CATCTACGCCTTCCGCATTG	2	Samaras & Pavlidis, 2018
	MC2R R		ATGAGCACCGCCTCCATT		
18s	18S F	KU820862	AGGAATTGACGGAAGGGCAC	2	Masroor et al., 2018
	18S R		TAAGAACGGCCATGCACCAC		
sod1	SOD1 F	DLA_LG14_005480	AACCATGGTGATCCACGAGA	1.9	Chang et al, 2021
	SOD1 R		ATGCCGATGACTCCACAGG		
sod2	SOD2 F	DLAgn_00071530	TGCCCTCCAGCCTGCTCT	1.7	Chang et al, 2021
	SOD2 R		CTTCTGGAAGGAGCCAAAGTC		
cat	CAT F	DLAgn_00171080	TGCTGAATGAAGAGGAGCGC	2	This study
	CAT R		ACAGCCTTCAAGTTCTGCAAC		
cs	CS F	DLAgn_00102430	TGGCGTCTATGAAAGTGTGG	1.9	This study
	CS R		CTGAAGTGAACATGGTGGCG		
ldh-a	LDHA F	DLAgn_00166080	TGACGCTGAGAACTGGAAGG	2	This study
	LDHA R		GTGCAGGTTCTTGAGGATGC		

	Body length (cm)	Body mass (g)	TL (*tel/mc2r*)	*tert*	*pcna*	*Casp8*	*casp9*	*sod1*	*sod2*	*cat*	*cs*	*ldh-a*
Body length (cm)	1,00											
Body mass (g)	0,96*	1,00										
TL (*tel/mc2r*)	0,26	0,18	1,00									
tert	0,16	0,18	0,48*	1,00								
pcna	0,23	0,31*	0,48*	0,31	1,00							
casp8	0,06	0,10	0,38*	0,64*	0,48*	1,00						
casp9	0,01	0,00	0,21	0,47*	0,28	0,78*	1,00					
sod1	0,00	0,15	-0,28	-0,05	0,52*	0,15	0,14	1,00				
sod2	0,24	0,23	0,18	0,66*	0,31	0,60*	0,57*	0,15	1,00			
cat	0,49*	0,44*	0,59*	0,26	0,28	0,18	0,06	-0,17	0,24	1,00		
cs	-0,31	-0,22	-0,58*	0,11	-0,09	0,45*	0,52*	0,12	0,34	-0,66*	1,00	
ldh-a	-0,30	-0,21	-0,39*	-0,12	-0,38*	-0,04	-0,13	0,02	-0,15	-0,26	0,48*	1,00

837 **Table 2** Spearman r-correlation matrix. Asterisks denotes significant r using $P < 0.05$.

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840 **Table 1S** Reproducibility of the "two-plate" telomere length assay using T/R ratio of 15
841 genomic DNA samples and one reference control in duplicates calculated using the ΔΔCt
842 method

	Sample	Reference	SW 1	SW 2	SW 3	SW 4	SW 5	FWt 1	FWt 2	FWt 3	FWt 4	FWt 5	FWi 1	FWi 2	FWi 3	FWi 4	FWi 5
	Assay # 1																
	Rep 1	1,04	1,06	1,07	0,97	0,79	1,08	1,09	0,84	1,07	0,96	0,98	0,81	0,93	1,03	0,86	0,90
	Rep 2	0,96	1,06	1,07	0,97	0,78	1,05	1,11	0,81	1,08	0,97	1,02	0,82	0,94	1,08	0,87	0,92
	Mean	1,00	1,06	1,07	0,97	0,79	1,06	1,10	0,83	1,08	0,97	1,00	0,82	0,94	1,06	0,86	0,91
	StDev	0,05	-	0,00	0,00	0,01	0,03	0,02	0,02	0,01	0,00	0,03	0,01	0,01	0,04	0,01	0,01
	CV %	5,39%	0,00%	0,00%	0,00%	0,98%	2,45%	1,47%	2,94%	0,98%	0,49%	2,94%	0,98%	0,98%	3,43%	1,47%	1,47%
																Intra-assay CV = 1,63%	
Reference gene: *mc2r*	**Assay # 2**																
	Rep 1	1,00	1,02	1,02	0,99	0,83	0,98	1,01	0,84	1,03	0,92	0,94	0,78	0,87	0,96	0,79	0,86
	Rep 2	1,00	1,08	1,00	0,98	0,83	1,00	1,10	0,87	1,08	0,93	1,00	0,82	0,94	1,01	0,83	0,89
	Mean	1,00	1,05	1,01	0,98	0,83	0,99	1,05	0,86	1,05	0,93	0,97	0,80	0,90	0,99	0,81	0,87
	StDev	0,00	0,04	0,01	0,01	0,00	0,01	0,06	0,02	0,03	0,00	0,05	0,02	0,04	0,03	0,03	0,03
	CV %	0,49%	3,43%	1,47%	0,98%	0,00%	1,47%	5,88%	1,96%	2,94%	0,49%	4,90%	2,94%	4,90%	3,43%	3,43%	2,94%
																Intra-assay CV = 2,63%	
																	Inter-assay CV = 2,13% (± 0,71%)
	Assay # 1																
	Rep 1	1,02	0,98	1,15		0,64	0,76	1,60	1,17	1,00	0,90			1,13	0,81	0,57	0,47
	Rep 2	0,98	1,01	1,27		0,66	0,84	1,65	1,16	1,01	0,92			1,17	0,79	0,57	0,48
	Mean	1,00	1,00	1,21		0,65	0,80	1,62	1,17	1,01	0,91			1,15	0,80	0,57	0,48
	StDev	0,03	0,02	0,09		0,02	0,05	0,03	0,01	0,01	0,01			0,03	0,01	-	0,01
	CV %	2,94%	2,45%	7,35%		2,45%	6,86%	1,96%	0,49%	0,98%	1,47%			2,45%	1,47%	0,00%	1,96%
																Intra-assay CV = 2,62%	
Reference gene: *18S*	**Assay # 2**																
	Rep 1	0,96	0,94	1,16		0,65	0,69	1,48	1,15	0,98	0,86			1,05	0,73	0,53	0,44
	Rep 2	1,04	0,96	1,14		0,67	0,70	1,60	1,17	1,02	0,89			1,11	0,76	0,53	0,47
	Mean	1,00	0,95	1,15		0,66	0,70	1,54	1,16	1,00	0,87			1,08	0,74	0,53	0,46
	StDev	0,05	0,01	0,01		0,01	0,01	0,08	0,01	0,02	0,02			0,04	0,02	0,01	0,02
	CV %	5,39%	0,98%	0,98%		1,96%	0,98%	5,39%	0,98%	2,45%	1,96%			3,43%	2,94%	0,98%	5,39%
																Intra-assay CV = 2,69%	
																	Inter-assay CV = 2,66% (± 0,05%)