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Relating Estradiol and Telomeres to Longevity in Marine Medaka *Oryzias melastigma*

雌二醇和端粒與青鱗魚 *Oryzias melastigma* 壽命的相關性研究

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ABSTRACT

Longevity gender gap (LGG) (females living longer than males) exists in humans and other animals. Ample evidence in mammals has shown that a gender difference exists in telomere length (TL) (females longer than males) and the rate of telomere attrition (females slower than males). Critical shortening of telomere may induce replicative senescence and ultimately lead to tissue stem cell exhaustion, age-related tissue degeneration, organismal aging and shortened lifespan. In mammals, estradiol (E2) has been proposed as a key factor responsible for reducing telomere attrition via two pathways: (1) increase telomerase activity (TA) to replenish telomeres loss, and/or (2) reduce oxidative damage to telomeric DNA. However, the relationships between E2, TL and longevity in non-mammalian vertebrates remain largely unexplored.

Marine medaka *Oryzias melastigma* possess a number of desirable characteristics for telomere and estrogen biology and longevity studies, including: (1) short TL (0.5 – 12 kb); (2) gradual senescence and telomere shortening with age; (3) known telomerase reverse transcriptase (TERT) and estrogen receptor-alpha (ERα) gene and protein sequences; (4) short lifespan (approximately 18 months), and available in large numbers under laboratory conditions; (5) estrogen biology similar to humans, and (6) distinct sexual dimorphism, the morphology of the anal fin is very

prominent in *O. melastigma* ca. 1 month after hatching, rendering it highly desirable for gender study.

In the present study, the *O. melastigma* was used as a model for studying the relationship between TL, E2 and longevity in non-mammalian vertebrates. The sex ratio and fish survival, plasma E2, testosterone levels of *O. melastigma* at 4 months (young), 8 months (middle-aged) and 12 months old (senior) were measured. Telomere length (by Southern blotting), telomerase activity (by RTQ-TRAP assay), gene expressions of telomerase reverse transcriptase (TERT) and ERα (by real time-PCR), DNA oxidation (by 8-OHdG) and protein oxidation (by protein carbonyl) were measured in the liver and gills of male and female *O. melastigma* at different ages.

The results show that LGG also exists in *O. melastigma* (females living longer than males). The female *O. melastigma* has begun to outnumber the males by the time they reach 10 months old. With advancing age, the proportion of short TRFs length (< 2 kb) increases in the liver of female and male *O. melastigma* and in the gills of female *O. melastigma*, showing a decline of TL with age.

In the liver, gender difference is clearly evident in TL, TA, TERT and ERα mRNA expressions in the young and middle-aged *O. melastigma*. Plasma E2 was positively correlated with liver ERα mRNA expression ($p \leq 0.001$) and liver TA ($p \leq$

0.05). In addition, liver ERα mRNA expression was positively correlated with liver TA ($p \leq 0.001$), suggesting up-regulation of TA by E2 in the liver was possibly mediated via ERα regulated pathways: TERT gene transcription and/or TERT protein phosphorylation. In parallel, liver DNA oxidation (as measured by 8-OHdG level) and oxidative protein damage (as measured by protein carbonyl level) were found to increase with age in *O. melastigma*, and the levels of oxidative damage were generally lower in females than in males. In addition, plasma E2 was inversely correlated with 8-OHdG ($p \leq 0.05$) and protein carbonyl content ($p \leq 0.001$), respectively, suggesting E2 may enhance cellular antioxidant defense in the liver, leading to a lower oxidative damage in the females. Collectively, in the liver, E2 may prevent telomere shortening by enhancing telomerase activity and reducing oxidation damage.

In the gills, gender difference in TL and TA were detected, but not for TERT mRNA expression. Given an absence of ERα mRNA expression in the gills, and a significant positive correlation between plasma E2 and gill TA ($p \leq 0.001$), the activity of telomerase in the gills of *O. melastigma* was unlikely to be mediated via TERT transcription, but likely to be induced by E2 via the TERT protein phosphorylation pathways. Moreover, the gill 8-OHdG and protein carbonyl levels were lower in middle-aged females than in males (8 months old), possibly because

females have stronger antioxidant defense capacity. When plasma E2 declined in old female *O. melastigma* (12 months), the antioxidant defense capacity in the female gills was weakened and the gender advantage faded out accordingly.

In conclusion, the results of this study provide a comprehensive understanding of the *in vivo* relationship between E2, TL and longevity in *O. melastigma.* The present findings support the view that E2 may also protect telomeres by increasing TA and reducing cellular oxidative stress and is also a key factor contributing to a greater longevity in female *O. melastigma*. Given the short generation time and distinct sexual dimorphism, *O. melastigma* may serve as a desirable alternative model for further studies of the possible mechanisms of gender-specific longevity in vertebrates.

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ACRONYMS AND ABBREVIATIONS

8-OHdG	8-hydroxydeoxyguanosine
µg	Micro-gram
µl	Micro-liter
µM	Micro-molar
ACX	Anchored return
AIDS	Acquired immunodeficiency syndrome
ANOVA	Analysis of variance
AP	Alkaline phosphatase
bp	Basepair
BSA	Bovine serum albumin
C	Cytosine
cDNA	Complementary DNA
CHAPS	3-[(3-Cholamidopropyl)-dimethyl-ammonio]-1-propane sulfonate
cm	Centi-meter
C_t	Threshold cycle
Cy3	Cyanine 3
DC	Dyskeratosis congenita
DDR	DNA damage responses
DIG	Digoxigenin
D-loop	Displacement loop
DNA	Deoxyribonucleic acid
Dnase	Deoxyribonuclease
dNTP	Deoxynucleoside triphosphate
DSB	Double-stranded DNA break
E2	17β-estradiol
EDTA	Ethylenediamine tetraacetic acid
EGTA	Ethyleneglycol-bis-(2-aminoethylether)-N,N,N',N' tetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
eNOS	Endothelial nitric oxide synthase
ER	Estrogen receptor
ERE	Estrogen responsive element
ERR	Estrogen-related receptor
F/M	Female / Male
FITC	Fluorescein isothiocyante
G	Guanine
g	Gram

GC	Gas chromatography
GPx	Glutathione peroxidase
h	Hour
H_2O_2	Hydrogen peroxide
HCl	Hydrochloric acid
HPLC	High pressure liquid chromatography
IGF-1	Insulin-like growth factor-1
IHC	Immunohistochemistry
ISH	*In situ* hybridization
kb	Kilobase
KCl	Potassium chloride
L	Liter
LC	Liquid chromatography
LGG	Longevity gender gap
M	Molarity
MAPK	Mitogen activated protein kinase
mg	Milli-gram
MgCl2	Magnesium chloride
mJ	Milli-joule
ml	Milli-liter
mM	Milli-molar
MNE	Mean normalized expression
MnSOD	Manganese-superoxide dismutase
mRNA	Messenger RNA
MS	Mass spectrometry
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NF_KB	Nuclear factor $_KB$
ng	Nano-gram
nm	Nano-meter
nM	Nano-molar
NO	Nitric oxide
nt	Nucleotide
NTC	No-template control
$O_2•–$	Superoxide anion
OD	Optical density
omER	*Oryzias melastigma* estrogen receptor
omERR	*Oryzias melastigma* estrogen-related receptor

omTERT	*Oryzias melastigma* telomerase reverse transcriptase
p	p-value, the probability of being false in concluding that there is a true difference among the samples
PCR	Polymerase chain reaction
PI3K	Phosphatidylinositol 3-kinase
PMSF	Phenyl-methyl-sulfonyl fluoride
POT1	Protection of telomeres 1
Q-FISH	Quantitative fluorescence in situ hybridization
r	Spearman correlation coefficient
Rap1	Repressor/activator-site binding protein 1
RIA	Radioimmunoassay
RNA	Ribonucleic acid
RNase	Ribonuclease
ROS	Reactive oxygen species
rpm	Round per minute
rRNA	Ribosomal RNA
RT	Reverse transcriptase
RTQ-TRAP	Real-time quantitative telomeric repeat amplification protocol
SDS	Sodium dodecyl sulphate
SIRT1	Sirtuin 1
SIRT2	Sirtuin 2
SSC	Saline-sodium citrate
T	Testosterone
TA	Telomerase activity
TAE	Tris-acetate-EDTA
TERT	Telomerase reverse transcriptase
TIFF	Tagged image file format
TIN2	TRF-1-interacting nuclear protein 2
TL	Telomere length
t-loop	Telomeric loop
TPP1	TIN2- and POT1-interacting protein 1
TR	Telomerase RNA
TRF	Telomere restriction fragment
TRF1	TTAGGG-repeat-binding factor 1
TRF2	TTAGGG-repeat-binding factor 2
TS	Telomerase substrate
U	Unit
UV	Ultra-violet

V	Volt
v/v	Volume by volume
WRN	Werner syndrome gene
WS	Werner's syndrome
γ-H2AX	Phosphorylated histone H2AX

CHAPTER 1 INTRODUCTION

1.1 Longevity gender gap

Longevity gender gap (LGG) is the longevity difference between the two sexes. Women having a higher life expectancy has been commonly reported in both the developed and developing countries from different parts of the world (Vina et al. 2003, 2005; Stindl 2004; Aviv et al. 2005; Vina and Borras 2010). According to the data obtained from 221 countries and regions published in The World Factbook, Central Intelligence Agency (CIA, USA) in 2010, women in 214 countries live longer than men for an average of 4.48 years (Appendix 1). Among the seven countries where men live longer, six of them were located in Southern Africa. Excess mortality associated with the acquired immunodeficiency syndrome (AIDS) has led to changes in the distribution of population by sex and age (The World Factbook, 2010). Epidemiological studies in Africa have shown that the infection rate of AIDS is sex- and age-specific, and was particularly high in women less than 30 years old (Quinn et al. 1986). Surveillance and seroprevalence studies in Eastern Africa, Uganda (Berkley et al. 1990) have shown that a high number of younger females were sexually exposed to AIDS-infected men, which has led to a lower life expectancy of women in this AIDS-prevalent country. In addition, the average life expectancy of people in these six African countries is 50.24 years which is much lower than that in the developed

countries (80.04 years) (The World Factbook, 2010), suggesting that mortality in those countries was in large part caused by factors other than age-related pathologies. In Montserrat, an island in the Caribbean Sea and the seventh country where men were reported living longer than women, the population size was only around 5,000 people and the life expectancy data was obtained after an estimated 8,000 residents left the island in July 1995, owing to the resumption of volcanic activity (The World Factbook). By eliminating those anomalous data obtained from countries where AIDS is prevalent or where the life expectancy figures were extrapolated from incomplete census (a total of 36 countries, including the seven countries where men live longer), it was revealed that in 2010, women live longer than men for a minimal of 1.89 years (in Equatorial Guinea) to the widest gap of 13.63 years (in Russia), even though more men were born than women in all countries (Appendix 1). In nearly all countries studied, women started to outnumber men once they reached middle age (Kinsella and Gist 1998; Stindl 2004; Aviv et al. 2005). Taken together, it is clear that LGG is common in both the developed and developing human societies.

In Hong Kong, from 1971 to 2010, life expectancy at birth was 5.3 – 7.7 years longer for women than for men, according to the figures provided by the Government of Hong Kong Census and Statistics Department, Demographic Statistics Section (Appendix 2). In the past four decades, the life expectancies at birth of both HK

women and men have kept rising to 10.6 and 12.2 years, respectively (Fig 1.1; appendix 2), mainly due to better healthcare and standard of living (Bartlett and Phillips 1995). However, the difference in gender gap remains steady over the years, regardless of the improvement of the quality of life (Fig 1.1; Appendix 2).

Fig 1.1 Life expectancy at birth by sex from 1971 to 2010 in Hong Kong.

Besides humans, many other species also exhibit a higher longevity in females than males, such as primates including chimpanzees (Allman et al. 1998; Hill et al. 2001), gorillas, orangutans, gibbons, spider monkeys (Allman et al. 1998) and sifakas (Richard et al. 2002), and other mammals such as rats (Asdell et al. 1967; Vina et al. 2003, 2005; Vina and Borras 2010), Soay sheep (Coulson et al. 2001), pilot whales and killer whales (Austad 1997). LGG (females living longer than males) has also

been reported in other vertebrates such as reptiles, e.g. lizards (Tinkle 1972, 1973; Ballinger and Congdon 1981; Strijbosch and Creemers 1988), amphibians, e.g. frogs (Ryan et al. 1983; Lauck 2005) and newts (Marzona et al. 2004; Grabherr et al. 2009), fish, e.g. spiny dogfish (Ketchen 1975), mosquitofish (Krumholz 1948; Vargas and de Sostoa 1996; Cabral and Marques 1999) and scaldfish (Castilho et al. 2003). LGG (females living longer than males) has also been documented in some invertebrates, for instance fruit flies (Lints et al. 1993; le Bourg and Minois 1996; Nuzhdin et al. 1997), medflies (Davies et al. 2005), butterflies (Scott 1974), mosquitoes (Liles and Delong 1960), seed beetles (Maklakov et al. 2007), ants (Holldobler and Wilson 1990; Keller and Jemielity 2006; Jemielity et al. 2007), bees (Keller and Jemielity 2006; reviewed in Austad 2006), tarantulas (reviewed in Austad 2006), tea red spiders (Das 1959), copepods (Wedekind and Jakobsen 1998) and the giant kidney-worms in the maned wolf (de Carvalho and Vasconcellos 1995). The above findings suggest that LGG is not limited to humans.

Five theories have been proposed in an attempt to explain the potential cause(s) of LGG, including: (i) males' unhealthy life style and risky behavior, (ii) random inactivation of the X chromosome, (iii) sexual size dimorphism, (iv) telomere length (TL) as well as (v) estrogen (Stindl 2004; Aviv et al. 2005), which will be discussed in detail in the following Sections 1.2 – 1.6.

1.2 Unhealthy life style and risky behavior of males

Unhealthy life style and risky behavior in males (including smoking and drinking habits) may result in a higher mortality rate in males (Emslie et al. 2002; Kruger and Nesse 2006; Phillips 2006). In Canada, accidents, injuries and suicides are the major cause of mortality for males before the age of 60 (Phillips 2006). However, the risky behavior of males cannot satisfactorily explain the difference in mortality rates of humans beyond the age of 60, at which the mortality rate of males is two times higher than that of females (reviewed in Stindl 2004). Unhealthy lifestyle is also a doubtful reason since, even though in the past 40 years the life style of females has become as unhealthy as that of males, the gender gap has widened (Table 1.1; Kinsella and Gist 1998; Stindl 2004). There have also been reports showing females living longer than males even when they have worse health conditions than males – known as the sex morbidity-mortality paradox (Nathanson 1975; Verbrugge 1982). In addition, because LGG can be observed in nearly all the developed and developing countries (Appendix 1) including HK (Appendix 2), it is therefore unlikely that it can be attributed solely to socio-demographic reasons (Vina and Borras 2010). Finally, because the longer lifespan of females over males is not specific to humans, but also occurs in other mammals (Borras et al. 2003; Vina et al. 2003, 2005; Austad 2006; Vina and Borras 2010), biological difference(s) between males and females would seem most likely

the reason for this discrepancy.

Table 1.1 Life expectancy at birth and LGG for selected countries. Data were extracted from Kinsella and Gist (1998).

Country	Circa 1900 (yr)			Circa 1950 (yr)			1998 (yr)		
	Female	Male	LGG	Female	Male	LGG	Female	Male	LGG
Australia	56.8	53.2	**3.6**	71.8	66.7	**5.1**	83	77	**6**
Austria	39.9	37.8	**2.1**	67	62	**5**	80.7	74.1	**6.6**
Belgium	48.9	45.4	**3.5**	67.4	62.1	**5.3**	80.7	74.1	**6.6**
Czech Republic	41.7	38.9	**2.8**	65.5	60.9	**4.6**	77.7	70.8	**6.9**
Denmark	54.8	51.6	**3.2**	71.5	68.9	**2.6**	79.1	73.6	**5.5**
France	48.7	45.3	**3.4**	69.4	63.7	**5.7**	82.6	74.6	**8**
Germany	46.6	43.8	**2.8**	68.5	64.6	**3.9**	80.3	73.8	**6.5**
Greece	39.7	38.1	**1.6**	66.7	63.4	**3.3**	81	75.8	**5.2**
Hungary	38.2	36.6	**1.6**	63.4	59.3	**4.1**	75.4	66.5	**8.9**
Italy	43.2	42.9	**0.3**	67.2	63.7	**3.5**	81.7	75.3	**6.4**
Japan	44.3	42.8	**1.5**	63.1	59.6	**3.5**	83.3	76.9	**6.4**
Norway	55.8	52.3	**3.5**	73.8	70.3	**3.5**	81.2	75.4	**5.8**
Spain	35.7	33.9	**1.8**	64.3	59.8	**4.5**	81.6	73.8	**7.8**
Sweden	55.3	52.8	**2.5**	72.6	69.9	**2.7**	82	76.5	**5.5**
United Kingdom	50.1	46.4	**3.7**	71.1	66.2	**4.9**	80.1	74.8	**5.3**
United States	51.1	48.3	**2.8**	71.7	66	**5.7**	79.6	72.9	**6.7**

Testosterone, a male steroid hormone, is associated with risky behavior and unhealthy lifestyles in men (Daltzman and Zuckerman 1980; Dabbs and Morris 1990; Booth and Osgood 1993; Mazur 1995; Booth et al. 1999; Blanco et al. 2001; Vermeersch et al. 2008). However, the effect of testosterone on longevity in men is controversial. Hamilton and Mestler (1969) found that the median lifespan in

castrated men, with a lower T level than normal men, was 13.6 years longer than that of intact men in a mentally retarded population. Conversely, a low serum testosterone level (less than 250 or 241 ng/dL, respectively) is associated with higher mortality rate in male veterans and elderly men (Shores et al. 2006; Laughlin et al. 2008). On the other hand, a study on data from encyclopedias and biographies reported there was no significant difference in lifespan between castrated and intact males born between 1581 and 1858 (Nieschlag et al. 1993). Therefore, there is no consensus on the effect of testosterone on men's lifespan.

1.3 Random inactivation of X chromosome

Every somatic cell of mammalian females contains two X sex chromosomes, one of which is inactivated stochastically in embryogenesis (for review, see Brown and Robinson 2000). Less than 25% of genes are transcribed on the inactivated X chromosome (Carrel and Willard 2005). Due to the random inactivation of X chromosome, females at their newborn contain equal amount of two types of somatic cells, each type contains an active X chromosome whereas the other one is inactivated (Christensen et al. 2000; Aviv et al. 2005). If one of the X chromosomes contains a disease-causing mutant gene, cells containing the active X chromosome with normal allele will have survival advantage and the mosaicism becomes unbalanced in favor of

cells with the normal X-linked gene to counter the harmful effect of the mutant gene on the hosts (Aviv et al. 2005). On the other hand, mammalian males' somatic cells contain one X and one Y sex chromosome, so males possess only one type of somatic cells (Christensen et al. 2000; Aviv et al. 2005). Therefore, males are susceptible to suffering from X-linked genetic diseases if they receive disease-causing X-linked genes from their mothers (Christensen et al. 2000; Aviv et al. 2005). Despite this, it is unlikely that mutations in genes on X chromosomes are all responsible for age-associated pathologies and that all mammalian males who receive those disease-causing mutant X-linked genes will have a short life expectancy (Stindl 2004). Table 1.2 shows a number of mammalian age-associated genes which are not located on the X chromosome.

Table 1.2 Locations of selected age-related genes on human chromosomes, extracted from National Center for Biotechnology Information (NCBI) (www.ncbi.nlm.nih/gene).

Age-related gene	Gene ID	Chromosome number in humans	Location on chromosome	Reference
Telomerase RNA (TR)	7012	3	3q26	Blasco et al. 1997; Lee et al. 1998; Herrera et al. 1999; Rudolph et al. 1999
Telomerase reverse transcriptase (TERT)	7015	5	5p15.33	Bodnar et al. 1998; Counter et al. 1998; Vaziri and Benchimol 1998
Werner syndrome gene (WRN)	7486	8	8p12	Reviewed in Fraga and Esteller 2007
Sirtuin 1 (SIRT1)	23411	10	10q21.3	Reviewed in Fraga and Esteller 2007
Sirtuin 2 (SIRT2)	22933	19	19q13	Reviewed in Fraga and Esteller 2007

1.4 Sexual dimorphism of size

Sexual size dimorphism refers to an observation that males are often larger in

body size than their female counterparts (Stindl 2004). A larger body size needs more doublings of the cell population for growth and maintenance, resulting in earlier stem cell exhaustion and faster aging (Stindl 2004). In addition, a larger body size has been found to be positively correlated to infectious diseases and age-related diseases such as cancer and male-biased mortality (Promislow 1992; Gunnell et al. 1998; Moore and Wilson 2002; Stindl 2004). However, a number of counter-examples can be found in nature. For instance, female spiny dogfish, mosquitofish, frogs, newts, ants, tea red spiders and the giant kidney-worms in the maned wolf have a larger body size and a longer lifespan than males (Krumholz 1948; Das 1959; Ketchen 1975; Ryan et al. 1983; de Carvalho and Vasconcellos 1995; Vargas and de Sostoa 1996; Cabral and Marques 1999; Marzona et al. 2004; Lauck 2005; Keller and Jemielity 2006). Given the controversy of this theory, it is doubtful whether sexual dimorphism of size is plausible in explaining LGG.

1.5 Longer telomere length (TL) in females

Ample evidence in mammals has shown that a gender difference exists in TL (females longer than males) and the rate of telomere shortening (females slower than males) (Coviello-McLaughlin and Prowse 1997; Jeanclos et al. 2000; Benetos et al. 2001; Cawthon et al. 2003; Cherif et al. 2003; Mayer 2006; Guan et al. 2007). In the

leukocytes of human newborns, there is no statistically significant difference in TL between males and females (Okuda et al. 2002). Cross-sectional population analyses have shown that the age-adjusted TL of pre-menopausal women is longer and erodes more slowly than that of similarly aged men (Jeanclos et al. 2000; Benetos et al. 2001; Cawthon et al. 2003; Mayer 2006; Guan et al. 2007). The findings suggest a gender difference in telomere erosion in humans (males > females).

In addition, the TL of females is approximately 240 bp longer than that of males (Jeanclos et al. 2000; Benetos et al. 2001; Brouilette et al. 2003; Wong and Collins 2003). The rate of telomere shortening in human leukocytes is ca. 30 bp per year (Slagboom et al. 1994; Jeanclos et al. 2000; Christensen et al. 2001). The calculated gender gap is 8 years (Aviv et al. 2005), which is within the observed LGG in human populations. Besides humans, gender difference in TL (females > males) has also been found in mice, rats and ants (Coviello-McLaughlin and Prowse, 1997; Cherif et al., 2003; Keller and Jemielity 2006; Jemielity et al. 2007), making TL a potential biological explanation for LGG. However, it remains largely unclear whether gender difference in TL exists in non-mammalian vertebrates and whether TL is a potential explanation for LGG in non-mammalian vertebrates.

1.5.1 Telomeres: structure and function

Telomeres are specialized DNA-protein complexes capped at the ends of linear eukaryotic chromosomes (Meyne et al. 1989; Blackburn 1991). In humans and other vertebrates, the telomeric DNA sequence consists of tandem repeats of 5'-TTAGGG-3', with a single-stranded G-rich 3' overhang, known as G-tail (Moyzis et al. 1988; Meyne et al. 1989; Blackburn 1991; Makarov et al. 1997; Wright et al. 1997). The telomere-binding protein complex in vertebtates, called shelterin, is composed of six components: TTAGGG-repeat-binding factor 1 (TRF1), TTAGGG-repeat-binding factor 2 (TRF2), repressor/activator-site binding protein 1 (Rap1), TRF-1-interacting nuclear protein 2 (TIN2), TIN2- and POT1-interacting protein 1 (TPP1) and protection of telomeres 1 (POT1) (Zhong et al. 1992; Bilaud et al. 1997; Broccoli et al. 1997; Kim et al. 1999; Li et al. 2000; Zhu et al. 2000; Baumann and Cech 2001; Houghtaling et al. 2004; Liu et al. 2004; Ye et al. 2004; Palm and de Lange 2008; O'Sullivan and Karlseder 2010). TRF1 and TRF2 bind double-stranded telomeric DNA, while POT1 attaches to single-stranded G-tail (O'Sullivan and Karlseder 2010). TIN2 joins TRF1 and TRF2, whereas TPP1 links TIN2 and POT1 (O'Sullivan and Karlseder 2010). The structure of mammalian telomere with shelterin is shown in Fig 1.2.

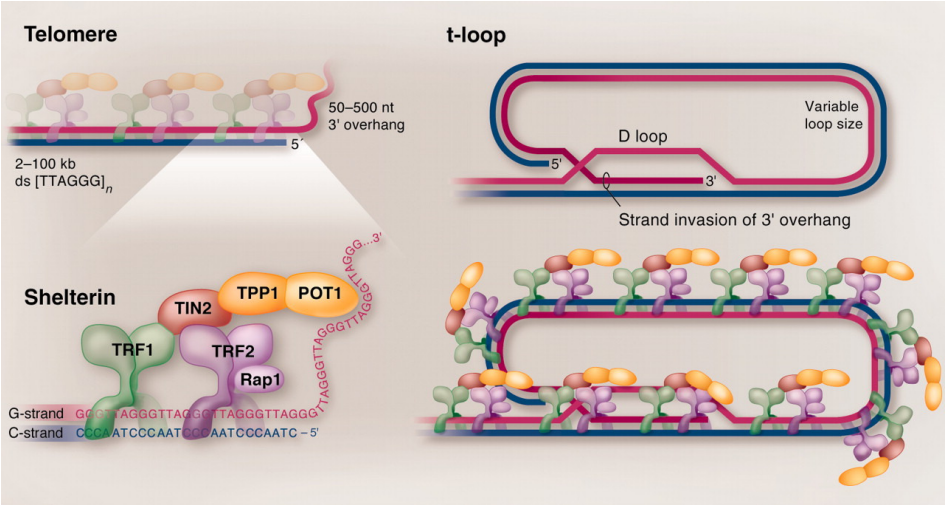


Fig 1.2 Structure of mammalian telomere (extracted from de Lange 2009).

Shelterin facilitates the formation of a telomeric loop (t-loop) and displacement loop (D-loop) structure which is held through the strand invasion of the G-tail into the duplex telomeric DNA repeat array (Greider 1999; Griffith et al. 1999). The t-loop and D-loop structure avoids the telomeric DNA to be mistaken as double-stranded DNA break (DSB) which will induce subsequent DNA damage responses (DDR) (de Lange 2005). In addition, shelterin negatively regulates the activity of telomerase by sequestrating the enzyme from its telomeric DNA substrate (de Lange 2005). As a whole, telomeres protect chromosome ends from degradation, end-to-end fusion and recombination (Garvik et al. 1995; Grandin et al. 1997, 2001; van Steensel et al. 1998). Therefore, the telomere-protein complex is a specialized structure for the maintainence of genome stability and chromosome integrity of the cell (Blackburn 1991; McEachern et al. 2000; Tsolou et al. 2008).

1.5.2 Telomere shortening, replicative senescence and organismal aging

Telomere shortening occurs with successive cell divisions in most of the eukaryotic cells as a result of the end replication problem (Watson 1972; Olovnikov 1973; Aviv et al. 2005) as well as oxidative damage to telomeric DNA (von Zglinicki et al. 1995; von Zglinicki 2002). The contribution of the end replication problem and oxidative damage to telomere shortening will be discussed in Sections 1.5.2.1 and 1.5.2.2, respectively. Cells of lower eukaryotes such as fungi and insects lose approximately 3 – 5 bp of telomeric DNA per cell division (Levis 1989; Lundblad and Szostak 1989; Johnson et al. 2001; Walter et al. 2001), whereas eukaryotic cells from vertebrates such as humans and mice shorten their telomeres with 50 – 200 bp in each round of DNA replication (Harley et al. 1990; Blasco et al. 1997; Niida et al. 1998).

1.5.2.1 End replication problem

The end replication problem occurs when conventional DNA polymerase only proceeds in the 5' → 3' direction and need a RNA primer of 8 – 12 nt to initiate the replication (for reviews, see Cong et al. 2002; Vega et al. 2003; Smogorzewska and de Lange 2004). The process is illustrated in Fig 1.3.

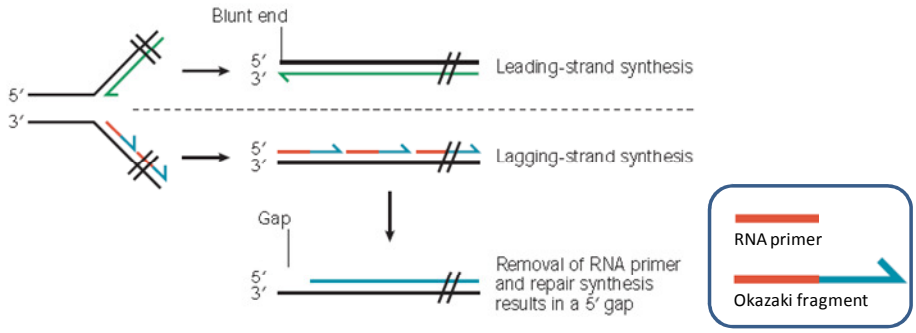


Fig 1.3 End replication problem (modified from Vega et al. 2003).

Short RNA-primed Okazaki fragments are synthesized on the lagging strand. The RNAs are then degraded by RNase and the gaps filled in by conventional DNA polymerase priming from upstream 3' end of the DNA fragment. When the 5'-most RNA primer is removed, an 8 – 12 nt 5' terminal gap is generated on the newly synthesized lagging strand. As a result, the extreme 5' end of DNA is progressively lost from the lagging strand each time a cell divides.

1.5.2.2 Oxidative damage to telomeric DNA

Reactive oxygen species (ROS) induces oxidative DNA damage leading to replicative senescence (Harman 2003). One of the most abundant products of oxidative DNA damage is 8-hydroxydeoxyguanosine (8-OHdG) (Tardieu et al. 1998; Helbock et al. 1999; Martinez et al. 2003, Evans et al. 2004). This is because guanine (G) is the most easily oxidized nitrogenous base (Nolan et al. 2003). The level of

8-OHdG increases with age in various organisms studied (Kregel and Zhang 2007). The fact that telomeric DNA contains G-rich tandem repeats (5'-TTAGGG-3') means that telomeres are particularly sensitive sites for ROS attack (Henle et al. 1999). This was exemplified by *in vitro* evidence, using human fibroblasts, where oxidative stress was shown to promote telomere shortening profoundly (von Zglinicki et al. 1995, 2000a; Vaziri et al. 1997; Saretzki et al. 1999). The efficiency of single-stranded break repair in the telomeric region was low (von Zglinicki et al. 2000a). Accumulation of single-stranded breaks resulted from oxidative DNA damage on telomeres before DNA replication (Petersen et al. 1998; Honda et al. 2001) and led to permanent loss of terminal single-stranded fragments available for DNA synthesis. Oxidative DNA damage accelerates telomere shortening (von Zglinicki et al. 1995, 2000a; Sitte et al. 1998; von Zglinicki 2000; Honda et al. 2001). In contrast, antioxidant enzymes were found to reduce the rate of telomere attrition and delay replicative senescence of fibroblasts (von Zglinicki et al. 2000b; Lorenz et al. 2001; Serra et al. 2003). This will be discussed in detail in Section 1.6.3.

1.5.2.3 Telomerase

Telomerase is a ribonucleoprotein enzyme for *de novo* telomere synthesis (Greider and Blackburn 1985, 1987; Morin 1989; Nugent and Lundblad 1998; Collins

and Mitchell 2002). In vertebrates, telomerase is composed of two sub-units: telomerase reverse transcriptase (TERT) (Lendvay et al. 1996; Lingner and Cech 1996; Lingner et al. 1997; Meyerson et al. 1997; Nakamura et al. 1997; Lingner and Cech 1998) and telomerase RNA (TR) (Greider and Blackburn 1989; Shippen-Lentz and Blackburn 1990; Feng et al. 1995; Chen and Greider 2004). TERT is a catalytic sub-unit with reverse transcriptase activity (Lingner et al. 1997; Meyerson et al. 1997; Nakamura et al. 1997; Lingner and Cech 1998), while TR serves as the RNA template for new telomeric DNA synthesis (Feng et al. 1995; Cech 2000). Through the repeated annealing of the RNA template of TR on telomere 3' overhang and nucleotide addition assisted with the catalytic activity of TERT, the G-tail of telomere will be elongated. With the newly extended G-tail acting as the template, the C-rich complementary strand is then synthesized by conventional DNA replication (for reviews, see Vega et al. 2003; Smogorzewska and de Lange 2004).

TR is ubiquitously expressed in both telomerase-positive and -negative cells (Avilion et al. 1996; Bryan et al. 1997; Yan et al. 2001). TERT, which is primarily regulated at the transcriptional and post-transcriptional levels (Meyerson et al. 1997; Nakamura et al. 1997; Anderson et al. 2006), modulates telomerase activity and, hence, telomere maintenance and cellular lifespan (Bodnar et al. 1998). In humans, telomerase activity is highly suppressed in most somatic cells, while the activity is

high in immortal cells such as embryonic stem cells, germ cells and cancer cells (Morin 1989; Kim et al. 1994; Wright et al. 1996; Shay and Bacchetti 1997; Cong et al. 2002; Flores et al. 2006). Telomerase-negative cells have limited replicative potential (Shay and Wright 2000; Wright and Shay 2001). On the other hand, in most unicellular eukaryotic organisms, telomerase is constitutively expressed to maintain telomere length (Meyerson et al. 1997; Nakamura et al. 1997). These findings indicate that the high level of telomerase in highly proliferative cells and tissues is essential to maintain TL and genomic stability.

1.5.2.4 Telomere shortening causes replicative senescence/apoptosis

When telomeres are critically shortened to a threshold level, the end of chromosome becomes uncapped, and subsequent DNA damage response and signaling pathways are activated to induce replicative senescence or apoptosis (Harley et al. 1990; Hara et al. 1991; Shay et al. 1991; Cherif et al. 2003; d'Adda di Fagagna et al. 2003; Takai et al. 2003; Zou et al. 2004; Garcia et al. 2007; Haussmann and Mauck 2008; Simm et al. 2008; for reviews, see Cong et al. 2002; Vega et al. 2003; Smogorzewska and de Lange 2004). The loss of telomere can be compensated or replenished by the enzyme telomerase (Greider and Blackburn 1985).

Progressive shortening of telomeres by length and rate is an important

mechanism in the aging process *in vitro* and possibly *in vivo* (de Lange et al. 1990; Harley et al. 1990; Hastie et al. 1990; Lindsey et al. 1991; Cherif et al. 2003; Garcia et al. 2007; Haussmann and Mauck 2008; Simm et al. 2008). It was shown that dividing human primary fibroblasts exhibited gradual telomere shortening until reaching the Hayflick limit (Harley et al. 1990). The Hayflick limit is the maximum number of times that normal mammalian somatic cells can divide *in vitro* (Hayflick and Moorhead 1961; Hayflick 1965). After approximately 50 – 70 doublings, a normal human somatic cell becomes senescent (Hayflick and Moorhead 1961). Further studies supported the speculation that telomere attrition limits cell proliferation in cell cultures (de Lange et al. 1990; Hastie et al. 1990; Lindsey et al. 1991). In immortal cells such as germ cells and cancer cells, their telomeres are maintained or elongated by the telomerase enzyme (Morin 1989; Kim et al. 1994). Ecotopic expression of TERT, which reconstitutes telomerase activity, was shown to maintain or elongate telomere length and immortalize normal somatic cells, e.g. fibroblasts and epithelial cells (Bodnar et al. 1998; Counter et al. 1998; Vaziri and Benchimol 1998; Jiang et al. 1999; Morales et al. 1999; Yang et al. 1999; Ouellette et al. 2000; Ramirez et al. 2001). Conversely, immortal cells induced or transformed with mutant TERT gene or under telomerase inhibition demonstrate telomere depletion and senescence phenotype (Lendvay et al. 1996; Counter et al. 1997; Hahn et al. 1999; Herbert et al. 1999;

Zhang et al. 1999). It has been clearly demonstrated that telomere shortening induces *in vitro* replicative senescence and limits cellular lifespan. Telomere shortening is believed to act as a mitotic clock, monitoring the replicative capacity of normal somatic cells (Harley et al. 1992; Wright and Shay 1992).

A number of *in vivo* findings have shown that TL declines with age in humans (blood, lymphocytes, abdominal aorta) (Vaziri et al. 1993; Okuda et al. 2000; Tsuji et al. 2002; Guan et al. 2007) and other vertebrates such as primates (baboons: granulocytes, B lymphocytes, T lymphocytes) (Baerlocher et al. 2003, 2007), mice (brain, cornea, hair follicles, skin, small intestine, spleen, testis) (Prowse and Greider 1995; Coviello-McLaughlin and Prowse 1997; Flores et al. 2008), rats (kidney, liver, lung, pancreas) (Cherif et al. 2003), cats (granulocytes, lymphocytes) (Brummendorf et al. 2002), birds (chicken: heart, intestine, kidney, lung, muscle; zebra finch, sand martins and dunlins: blood) (Taylor and Delany 2000; Haussmann and Vleck 2002; Pauliny et al. 2006), reptiles (American alligator: erythrocytes) (Scott et al. 2006) and fish (Japanese medaka: gonad, heart, intestine, kidney, liver, muscle; wild-derived strain of turquoise killifish: muscle, skin; zebrafish: whole fish) (Hatakeyama et al. 2008; Hartmann et al. 2009; Anchelin et al. 2011). These findings show that telomere shortening with age occurs not only *in vitro* but also *in vivo*.

Ample evidence from mammalian studies indicate that replicative senescence or

apoptosis compromises tissue homeostasis and function that may eventually lead to organismal aging (Chen et al. 2007; Simm et al. 2008; for review, see Nalapareddy et al. 2008). The inverse correlation between cellular replicative potential and donor age is well established in humans (Martin et al. 1970, for review, see Campisi 2001). Moreover, in humans, other mammals and tortoises, the *in vitro* proliferative capacity has been positively correlated with the maximum longevity of the animals from which the cells were derived (Goldstein 1974; Stanley et al. 1975; Rohme 1981). Furthermore, senescent cells accumulate with advancing age in human vascular tissue (Minamino et al. 2004; Matthews et al. 2006; Minamino and Komuro 2007), human corneal endothelium (Mimura and Joyce 2006), human retinal pigment epithelium (Hjelmeland et al. 1999), human and primate skin (Dimri et al. 1995; Pendergrass et al. 1999; Herbig et al. 2006; Jeyapalan et al. 2007), human and rat kidney (Ding 2001; Melk et al. 2003, 2004) and almost all tissues studied in mice (Krishnamurthy et al. 2004), indicating a reduction of replicative cells in aging tissues. A decrease in replicative cells in tissues may compromise tissue maintenance/repair and hinder organ function. Finally, cells derived from individuals with premature aging syndromes exhibited diminished *in vitro* replicative capacity (Martin et al. 1970; Salk et al. 1981; Martin 1982; Faragher et al. 1993; Davis et al. 2007; for review, see Chen et al. 2007), suggesting that the proliferative potential of cells may be related to the

aging of the host. On the other hand, apoptotic cells are prevalent in aging tissues of the human brain (Guo et al. 2004), heart (Phaneuf and Leeuwenburgh 2002), pancreas (Maedler et al. 2006) and T cell subsets ($CD4^+$ and $CD8^+$ T cells; Aggarwal and Gupta 1998). Apoptotic cells are also accumulated in aging rat heart (Phaneuf and Leeuwenburgh 2002), liver (Ando et al. 2002), gastric mucosa (Tarnawski et al. 2007) and muscle (Tamilselvan et al. 2007), as well as mouse heart, liver, muscle, testis (Kujoth et al. 2005) and rodent chondrocytes of articular cartilage (Adams and Horton 1998). Taken together, both senescent and apoptotic cells accumulate in mammalian tissues with age. The reduction of replicative cells in tissues may compromise tissue homeostasis and function, resulting in organismal aging. However, the relationship between telomere shortening and replicative senescence/apoptosis in non-mammalian vertebrates still remains unclear.

1.5.2.5 Telomere shortening and organismal aging

Replicative senescence or apoptosis induced by telomere shortening may ultimately lead to tissue stem cell depletion, age-associated tissue degeneration, organismal aging and shortened lifespan (Chin et al. 1999; Rudolph et al. 1999; Dokal 2001; Vulliamy et al. 2001; Cawthon et al. 2003; Chang et al. 2004, for review, see Baird 2008; Sahin and DePinho 2010). For humans over 60 years old, shorter

telomeres (bottom 25% of telomere length distribution of participants ranged from 1930 – 4310 bp) in peripheral blood leukocytes may be predictive of poor health conditions and diminished lifespan (3.18-fold higher mortality rate from heart disease, 8.54-fold higher mortality rate from infectious disease and nearly 2-fold higher overall mortality rate for individuals with shorter telomeres) (Cawthon et al. 2003). In line with this finding, a human population study on Ashkenazi Jewish centenarians reported that a significantly longer TL in leukocytes of Jewish centenarians than age-adjusted controls (humans of approximately 70 years old) was associated with a better health profile (better cognitive function, lipid profiles and protection from age-associated diseases including hypertension, metabolic syndrome and diabetes) and exceptional longevity (Atzmon et al. 2010). A significantly shorter TL in human peripheral blood mono-nuclear cells among participants (patients and controls) is also associated with a number of age-associated diseases, including atherosclerosis (Samani et al. 2001; Benetos et al. 2004), myocardial infarction (Brouilette et al. 2003; Fitzpatrick et al. 2006), stroke (Fitzpatrick et al. 2006), dementia (von Zglinicki et al. 2000b; Honig et al. 2006; Martin-Ruiz et al. 2006) and Alzheimer's disease (Panossian et al. 2003). Moreover, compromised telomere function is associated with age-related degenerative conditions and premature aging syndromes such as dyskeratosis congenita (DC) and Werner's syndrome (WS) (Dokal 2001; Vulliamy et

al. 2001; Chang et al. 2004). DC is caused by the mutation of TERT, TR or dyskerin, a TR binding protein responsible for TR stability and telomerase assembly, leading to telomere dysfunction and rapid telomere shortening (Mitchell et al. 1999; Wang and Meier 2004; Kirwan and Dokal 2009). WS is caused by the mutation of WRN helicase, resulting in telomere maintenance defects (Kruk et al. 1995; Schulz et al. 1996). Patients suffering from these degenerative disorders have reduced telomeres, accelerated aging and shortened lifespan (Dokal 2001; Vulliamy et al. 2001; Chang et al. 2004; Martin 2005). In addition, TERT transgenic mice engineered to be cancer-resistant by enhanced expression of p53/p16/p19ARF have significantly longer telomeres in their stem cell compartments in hair bulge, interfollicular epidermis and intestinal villi, longer medium lifespan and more youthful profile as indicated by improved neuromuscular coordination, higher glucose tolerance and serum insulin-like growth factor-1 (IGF-1) level, decreased phosphorylated histone H2AX (γ-H2AX) foci (a biomarker of DNA DSBs) and age-related pathologies, including degenerative inflammatory pathologies of skin and gastrointestinal tract, compared to age-matched controls (Tomas-Loba et al. 2008). On the other hand, telomerase-deficient mice with the depletion of TR show telomere attrition (telomere shortening rate: 4.8 ± 2.4kb per $TR^{-/-}$ mouse generation) leading to tissue degenerative phenotypes (as indicated by hair graying, alopecia, skin lesions, delayed wound

healing, depletion of male germ cells, diminished haematopoiesis and impaired mitogen-induced primary splenocyte proliferation) and shortened lifespan ($TR^{-/-}$ mouse of third-generation or beyond) (Blasco et al. 1997; Lee et al. 1998; Herrera et al. 1999; Rudolph et al. 1999). Mice engineered with human degenerative disorder mutations such as ATM-null, WRN-null or DMD-null show no classical premature aging pathologies unless their telomeres are depleted by telomerase knockout (Wong et al. 2003; Chang et al. 2004; Sacco et al. 2010). Intriguingly, reactivation of TERT in aged (telomerase-deficient) mice can extend their telomeres and reverse age-related tissue atrophy across highly proliferative organs including intestines, spleens and testes (Jaskelioff et al. 2011). However, the relationship between telomere shortening and aging is solely based on mammalian models. The role of TL on aging in non-mammals is virtually unknown. Whether TL is a good explanation for LGG (females live longer than males) in non-mammalian vertebrates is still unclear.

1.6 Higher estrogen level in females

Estrogen is a female steroid hormone that modulates the expression of a myriad of genes and proteins in estrogen responsive tissues and organs (for reviews, see Zhu and Conney 1998; Bayne et al. 2007). Abundant evidence suggests this hormone may be responsible for the observed LGG in humans and other mammals through the

reduction of telomere shortening (Aviv 2002; Borras et al. 2003, 2007; Vina et al. 2003, 2005; Aviv et al. 2005; Mayer 2006; Bayne et al. 2007; Guan et al. 2007; Vina and Borras 2010), which will be discussed in detail in Section 1.6.1.

In vitro studies using mammalian cell lines have shown 17β-estradiol (E2), the predominant form of estrogen in non-pregnant females, inhibits replicative senescence of human endothelial progenitor cells (as measured by acidic β-galactosidase staining) (Imanishi et al. 2005). Conversely, inhibition of E2 by transfecting dominant negative mutant gene of ERα into K-Ras-mediated NIH3T3 cells induced replicative senescence (as determined by senescence-associated β-galactosidase staining), showing an increase in the number of senescent cells, and flow cytometry with propidium iodine staining, demonstrating that the percentage of cells accumulating in the G_0/G_1 phase increased (Kato et al. 2002). Female mice with deficient of aromatase, an enzyme responsible for the conversion of androgen into estrogen (Fisher et al. 1998; Bayne et al. 2008), or mutant in ERα gene (Lubahn et al. 1993) suffer from atrophic changes in a number of estrogen responsive organs such as uteri and ovaries. Taken together, it is likely that estrogen avoids replicative senescence of mammalian estrogen-targeted cells through the prevention of telomere erosion.

1.6.1 Estrogen and telomeres

Estrogen may be a key factor contributing to the observed gender difference in TL, rate of telomere erosion and aging in humans (Aviv 2002; Borras et al. 2003, 2007; Vina et al. 2003, 2005; Aviv et al. 2005; Mayer 2006; Bayne et al. 2007; Guan et al. 2007; Vina and Borras 2010). Interestingly, the increasing rate of telomere attrition in women after menopause (Guan et al. 2007) and the attenuation of telomere erosion in post-menopausal women having taken estrogen for more than five years (Lee et al. 2005) further substantiate the involvement of estrogen in mediating gender difference in TL.

In addition, aromatase-knockdown female mice, suffering from estrogen deficiency, exhibited a down-regulation of TERT, inhibition of telomerase, attrition of telomeres and arrest of cell proliferation in the adrenal gland (an estrogen responsive organ), whereas administration of estrogen to aromatase-knockdown female mice for three weeks restored TERT mRNA expression, telomerase activity and cell proliferation (Bayne et al. 2008). Estrogen may reduce the rate of telomere attrition via two mechanisms: (1) an increase of telomerase activity (Section 1.6.2), and/or (2) a reduction of oxidative damage to telomeric DNA (Section 1.6.3).

1.6.2 Estrogen (E2) increases telomerase activity (TA)

Mounting *in vivo* evidence in mammals indicates that telomerase activity is activated by estrogen in endometrium, ovarian, breast, prostate and other estrogen-regulated tissues (Meeker et al. 1996; Kyo et al. 1997, 1999; Saito et al. 1997; Bednarek et al. 1998; Tanaka et al. 1998; Kang et al. 1999; Takakura et al. 1999; Misiti et al. 2000; Breitschopf et al. 2001; Williams et al. 2001; Nanni et al. 2002; Kawagoe et al. 2003; Du et al. 2004; Kimura et al. 2004; Imanishi et al. 2005; Boggess et al. 2006; Ling et al. 2006; Sarkar et al. 2006; Bayne et al. 2008; Cha et al. 2008).

Three mechanisms have been proposed to explain E2-mediated up-regulation of telomerase activity in ER-positive cells in mammals: (a) via an up-regulation of TERT mRNA expression mediated by transcription factor E2-ER complexes or c-myc (Kyo et al. 1999; Misiti et al. 2000; Nanni et al. 2002; Kawagoe et al. 2003; Kimura et al. 2004; Imanishi et al. 2005; Boggess et al. 2006; Sarkar et al. 2006; Bayne et al. 2008; Cha et al. 2008), (b) via an up-regulation of TERT mRNA expression mediated by nitric oxide (NO) stimulation (Vasa et al. 2000; Aviv 2002; Rahimian et al. 2004; Zaccagnini et al. 2005; Grasselli et al. 2008), and/or (c) via a post-transcriptional phosphorylation of TERT protein (Kang et al. 1999; Breitschopf et al. 2001; Kawagoe et al. 2003; Du et al. 2004; Kimura et al. 2004; Imanishi et al. 2005). Each of the

above mechanisms will be discussed in detail in following Sections (1.6.2.1 – 1.6.2.3).

1.6.2.1 E2 up-regulation of TERT gene transcription by E2-ER complexes or c-myc

E2 activates telomerase activity in ER-positive cells and estrogen-responsive tissues through an induction of TERT gene transcription either directly by transactivation of TERT promoter by E2-ER complexes (Fig 1.4) or indirectly by up-regulation of c-myc (Fig 1.5) (Kyo et al. 1999; Misiti et al. 2000; Nanni et al. 2002; Kawagoe et al. 2003; Kimura et al. 2004; Imanishi et al. 2005; Boggess et al. 2006; Sarkar et al. 2006; Cha et al. 2008; for reviews, see Cong et al. 2002; Bayne and Liu 2005; Bayne et al. 2007, 2008).

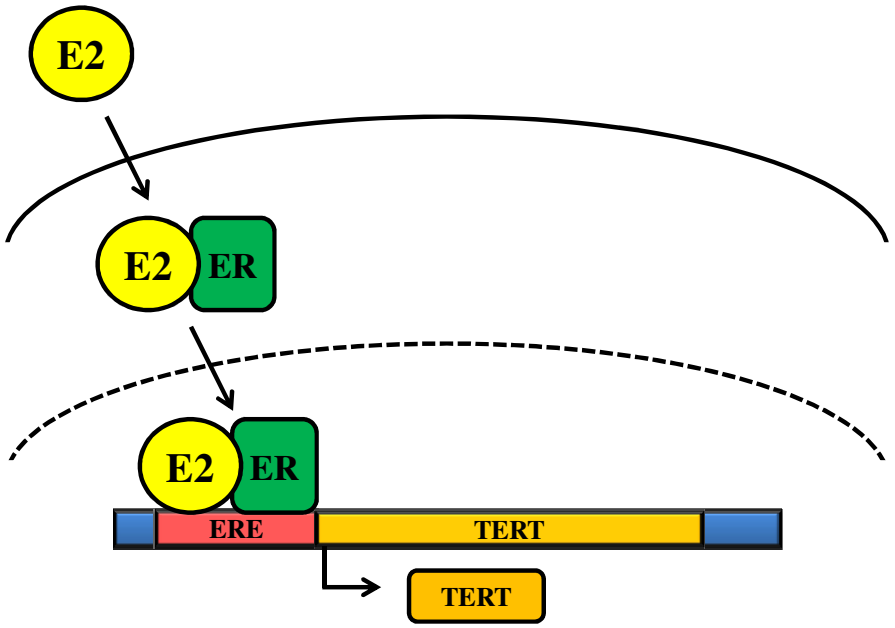


Fig 1.4 Estrogen mediated TERT transactivation via the E2-ER complex.

In human breast cancer cells, ovary epithelium cells and mesenchymal stem cells, E2 combines with ERα but not ERβ to form E2-ERα complexes which then bind to estrogen responsive elements (EREs) localized in the 5'-flanking region of the TERT gene, transactivate TERT promoter, induce TERT gene transcription and telomerase activity (Kyo et al. 1999; Misiti et al. 2000; Cha et al. 2008). In human prostate normal epithelial and cancer cells, telomerase activity is modulated under the same transcriptional regulation, whereas both ERα- and ERβ-E2 complexes can transactivate the TERT promoter (Nanni et al. 2002).

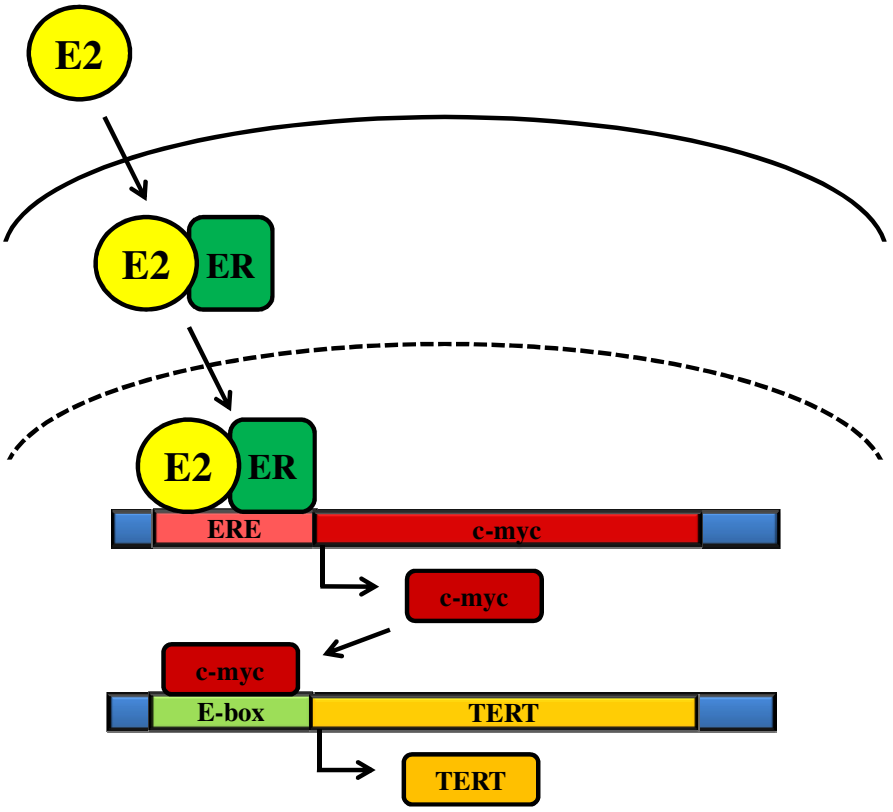


Fig 1.5 Estrogen mediated TERT transactivation via c-myc.

The promoter of c-myc gene contains EREs and c-myc is inducible by E2 (Kyo et al. 1999; Sarkar et al. 2006). The c-myc protein can bind to E-boxes located in the TERT promoter, and transactivate TERT gene expression (Kyo et al. 1999; Sarkar et al. 2006). Both the direct (via the E2-ERα complexes) and indirect (via c-myc) pathway of TERT transactivation by E2 have been reported in human breast cancer cells and rat fibroblasts (Kyo et al. 1999; Sarkar et al. 2006).

1.6.2.2 E2 Up-regulation of TERT gene transcription via nitric oxide (NO) stimulation

E2 stimulates telomerase activity via NO generation (Vasa et al. 2000; Aviv 2002; Rahimian et al. 2004; Zaccagnini et al. 2005; Grasselli et al. 2008). Vasa et al. (2000) reported that in aging human endothelial cells (after 11 passages), upon repeated addition of an NO donor (e.g. S-nitroso-penicillamine), age-related decline of telomerase activity, telomere shortening and replicative senescence were reduced, whereas the addition of an endogenous NO synthesis inhibitor (e.g. N^G–monomethyl-L-arginine) repressed telomerase activity and induced replicative senescence. Endogenous NO production in human endothelial cells *in vitro* is stimulated by the endothelial nitric oxide synthase (eNOS). The expression and activity of eNOS are inducible by E2 (Rahimian et al. 2004; Grasselli et al. 2008) and ERα (Chen et al. 1999; Chambliss and Shaul 2002; Grasselli et al. 2008). *In vitro* and *in vivo* studies on mice have shown NO increases TERT gene expression, telomerase activity and cell proliferation (Chen et al. 2005; Zaccagnini et al. 2005), however, the findings were exclusively based on endothelial cells as the model system (for review, see Zeng and Xu 2008). The mechanism(s) by which NO activates, directly or indirectly, TERT mRNA transcription in non-endothelial cells and tissues still largely remains obscure.

1.6.2.3 Post-translational phosphorylation of TERT protein

In mammalian cells E2 increases telomerase activity by TERT protein phosphorylation through the phosphatidylinositol 3-kinase (PI3K)/Akt pathway and/or PI3K/Akt/nuclear factor $_{K}$B ($NF_{K}B$) pathway (Kang et al. 1999; Breitschopf et al. 2001; Kawagoe et al. 2003; Du et al. 2004; Kimura et al. 2004; Imanishi et al. 2005; for reviews, see Cong et al. 2002; Kyo and Inoue 2002; Bayne et al. 2007). E2, forming complexes with ERα but not ERβ, interacts with PI3K (Simoncini et al. 2000) and transiently phosphorylases and activates Akt in mammalian cells (Hisamoto et al. 2001; Du et al. 2004; Cha et al. 2008). *In vitro* studies showed that activated Akt kinase could phosphorylase TERT and enhance telomerase activity (Kang et al. 1999; Breitschopf et al. 2001; Du et al. 2004; Kimura et al. 2004). E2 also enhances the association of TERT with $NF_{K}B$ p65 which is responsible for the nuclear translocation of TERT (Akiyama et al. 2003; Kawagoe et al. 2003; Du et al. 2004; Kimura et al. 2004). E2-induced phosphorylation of TERT protein enhances telomerase activity in mammalian cells.

1.6.3 Estrogen reduces oxidative damage to telomeric DNA

Oxidative stress occurs when the levels of ROS exceed the capacity of the antioxidant defense system, and results in oxidative damage to DNA, proteins and

lipids (Sastre et al. 1996; Marnett 2000). Cellular ROS formation can be stimulated by external inducers such as ultraviolet radiation, or during normal cellular metabolism, in particular mitochondrial respiration (for review, see Rattan 2006). Over 90% of oxygen consumed in mammalian cells is used up in mitochondria for cellular respiration. However, not all O_2 forms H_2O as the end-product, approximately 1–2% forms superoxide anion ($\mathbf{O_2^{\bullet -}}$) instead (Vina et al. 2003, 2005). Superoxide can be converted into H_2O_2 either spontaneously or catalyzed by the enzyme MnSOD (manganese-superoxide dismutase) in mitochondria (Vina et al. 2003, 2005). Mitochondrial H_2O_2 can leak into the cytoplasm (Forman and Azzi 1997) and be converted into H_2O by GPx (glutathione peroxidase) and/or catalase (for review, see Wassmann et al. 2004). Sohal and Weindruch (1996) have demonstrated that ROS production is negatively correlated with the maximum lifespan of Drosophila, whereas a high antioxidant defense capacity extends the lifespan of mammalian species (Barja and Herrero 2000). Moreover, long-lived species tend to produce a lower level of ROS compared to that of the short-lived species (Sohal et al. 1990; Ku et al. 1993).

Oxidative DNA damage in human leukocytes is lower in females than in males, as indicated by the level of 8-OHdG which is a sensitive and stable biomarker for DNA damage induced by ROS (Chen et al. 1999). The level of 8-OHdG in female

urinary excretion is also lower than that in males (Loft et al. 1992), indicating a gender difference in oxidative DNA damage in humans.

Telomeres are especially susceptible to oxidative damage because telomeres contain guanine (G)-rich sequence (Moyzis et al. 1988; Meyne et al. 1989; Blackburn 1991; Makarov et al. 1997; Wright et al. 1997) (see detail in Section 1.5.2.2) and guanine is the most easily oxidized base (Nolan et al. 2003). It has been found that the amount of oxidative DNA damage as determined by 8-OHdG is positively correlated with the rate of telomere shortening in human WI-38 fibroblasts (Oikawa et al. 2001).

Estrogen has been proposed as a factor contributing to less oxidative DNA damage in females (Vina and Borras 2010). In Wistar rats, the liver and brain mitochondria of females manufactured 50% less H_2O_2 and exhibited higher gene expressions and enzyme activities of MnSOD and GPx than those of male rats (Borras et al. 2003; Vina et al. 2003, 2005; Vina and Borras 2010). When the female rats were ovariectomized for 1 month, the levels of H_2O_2 production in liver and brain mitochondria rose to the level found in male rats, whereas when the female ovariectomized rats were treated with E2, the H_2O_2 level was reduced to that of the control females (Vina et al. 2003, 2005; Vina and Borras 2010). Interestingly, when young ovaries were transplanted to old, ovariectomized females, the longevity of the transplanted female mice was extended (Cargill et al. 2003; Mason et al. 2009).

Furthermore, in human breast cancer cell line, the addition of E2 significantly reduced the level of H_2O_2, while the simultaneous addition of ER antagonist abolished the effect (Vina et al. 2003, 2005). Taken together, estrogen reduces ROS production and oxidative damage to DNA, especially the telomeric DNA. The antioxidant defense capability of estrogen can be explained by two possible reasons: (a) estrogen as a chemical antioxidant (Ruiz-Larrea et al. 1997) and/or (b) estrogen induces the expression and activity of antioxidant enzymes (Borras et al. 2003; Vina et al. 2003, 2005; Vina and Borras 2010), which will be evaluated in the following Sections (1.6.3.1 – 1.6.3.2).

1.6.3.1 Estrogen as chemical antioxidant

Estrogen acts as a chemical antioxidant *in vitro* due to its phenolic structure containing phenolic hydroxyl groups which can donate proton to oxidants (Ruiz-Larrea et al. 1997). Nevertheless, the beneficial effect of estrogen on the reduction of oxidative stress is unlikely limited to its antioxidant property (Vina et al. 2003, 2005; Vina and Borras 2010). According to the calculation of Vina et al. (2005), the recommended dose of E2 intake for estrogen replacement therapy is 50 μg/day, whereas the recommended dose of vitamin E, being a more powerful antioxidant than E2, is 500 mg/day – x10,000 higher than that of E2. Given the very low physiological

level of E2 as an effective chemical antioxidant, it has been proposed that the ability of E2 to repress oxidative damage inside cells could be enhanced by the induction of antioxidant genes expressions and enzyme activities (Vina et al. 2003, 2005; Vina and Borras 2010).

1.6.3.2 Up-regulation and activation of antioxidant enzymes

It has been demonstrated that E2 binding to ER (estrogen receptor) activates the MAPK (mitogen activated protein kinase) and NFκB (nuclear factor kappa B) signaling pathway, leading to transcriptional up-regulation of MnSOD and GPx (Vina et al. 2003). In rats, females exhibited a higher antioxidant gene expression and lower oxidative damage than males (Borras et al. 2003). It has been well-documented that oxidative stress increases telomere attrition and induces replicative senescence *in vitro* (Petersen et al. 1998; Henle et al. 1999; Oikawa et al. 2001; von Zglinicki 2002; Kurz et al. 2004). It has been speculated that up-regulation of the antioxidant enzyme genes (MnSOD and GPx) by E2 can reduce ROS mediated oxidative damage to telomeric DNA, resulting in a decrease in the telomere attrition rate and a delay in replicative senescence, and hence a slow aging process and long longevity in females (Vina et al. 2005; Tarry-Adkins et al. 2006). Despite some reports indicating that estrogen replacement therapy is linked to an increase in life expectancy of postmenopausal

women (Ettinger et al. 1996; Paganini–Hill et al. 2006), to date there is no direct evidence supporting the view that E2 increases longevity *in vivo*. Study of the effects of E2 on TL and longevity *in vivo* is complicated by the fact that E2 increases the risk of ovarian and breast cancers in females and induces feminization of males (Clemons and Goss 2001; Oettel 2002; Sayed and Taxel 2003; Rohan et al. 2008; Spillman et al. 2010). A non-mammalian model exhibiting estrogen physiology, telomerase and telomere regulation would be a desirable alternative model for studying the *in vivo* effects of estrogen on TL and the link of E2 and TL to organismal aging and longevity. A thorough study of estrogen on aging phenotypes and longevity of non-mammalian vertebrate models would shed light on the potential role of estrogen on gender difference in telomere attrition and longevity in human/vertebrates.

1.7 Limitations of common model organisms for telomere and aging studies

Budding yeast (*Saccharomyces cerevisiae*), worms (*Caenorhabditis elegans*), flies (*Drosophila melanogaster*) and mice (*Mus musculus*) are widely-used model organisms for biological study, including telomere studies. The reason for this is that these four species are among the few organisms about which scientists have a wealth of knowledge relating to their genomes, heredity, chromosome structure, life history, development, maturation, physiology and reproduction, as well as stock centers

maintaining wild-type and mutant strains, and bio-techniques, tools and reagents such as antibodies and cell lines specially for scientific research on them (Arking 2006). Table 1.3 shows the number of published journals for telomere biology based on these four model organisms, together with humans, searched on PubMed, NCBI, indicating that these four species are prevalent models in this field.

Table 1.3 The number of published journals for telomere biology using humans, mice, flies, worms, budding yeast as the model organisms. Data obtained from PubMed, NCBI (www.ncbi.nlm.nih.gov/pubmed) dated April 8, 2011.

Common name	Binomial name	No. of journals
Humans	*Homo sapiens*	7,779
Mice	*Mus musculus*	1,536
Flies	*Drosophila melanogaster*	219
Worms	*Caenorhabditis elegans*	87
Budding yeast	*Saccharomyces cerevisiae*	1,476

However, these four model organisms have their own limitations as good models in telomere and aging research. Budding yeast is a single-cell eukaryote, making it an undesirable model for the investigation of the aging process for multicellular organisms. Worms and flies have a different anatomical organization from vertebrates (Terzibasi et al. 2007), making them not as desirable as a vertebrate model. Secondly,

their genetic regulation of aging is much simpler than that of vertebrates (Kishi et al. 2008). Thirdly, postmitotic cells are predominant in the somatic tissues of adult worms and flies, making it unfeasible to investigate replicative senescence (Terzibasi et al. 2007).

Mice, as a major mammalian model for biological studies, share the same telomeric sequence as humans (Moyzis et al. 1988; Meyne et al. 1989). However, the extraordinarily long TL of mice (50 – 150 kb) is a disadvantage for any investigation of the effects of telomere shortening on aging in short-term experiments (Blasco et al. 1997). For the telomerase knockout mice to display telomere-dependent aging phenotypes, the transgenic mice are bred for three generations to allow their telomeres to be eroded to a critical threshold for the emergence of age-related phenotypes (Blasco et al. 1997; Lee et al. 1998; Herrera et al. 1999; Rudolph et al. 1999). Clearly, studies using alternative vertebrate models with TL similar to that in humans (10 – 15 kb), and with established estrogen-related molecular and cellular biology, would be more instructive in unravelling the effects of E2 on telomeres, telomerase and antioxidant enzymes *in vivo* and shedding light on the gender-specific telomere biology and aging in humans.

1.8 Medaka as good model organism for telomere and aging studies

Since the 1960s, small laboratory model fish have been increasingly employed as vertebrate models in a variety of biomedical research (Bolis et al. 2001; Wakamatsu et al. 2001). The progression of aging in fish shares a number of common hallmarks with higher vertebrates such as increasing mortality rate with age and the possession of age-related biochemical and pathological phenotypes similar to those in mammals (Patnaik et al. 1994; Bolis et al. 2001; Gerhard 2007; Terzibasi et al. 2007; Ding et al. 2010). Small fish are generally easy to maintain and breed, amenable to laboratory conditions, have a short generation time (2 – 3 months for sexually mature) and propagate in large numbers, making them convenient for studying a wide range of developmental, reproductive and aging endpoints within a relatively short time and with statistical precision.

The marine medaka *Oryzias melastigma* was chosen as the model organism for this study among other widely-used models and the commonly-used small fish models, because marine medaka *O. melastigma* possess a number of desirable characteristics as a good model for telomere and estrogen biology and aging studies, including: (1) short TL (0.5 – 12 kb); (2) gradual senescence and telomere shortening with age; (3) known TERT and ERα gene and protein sequences; (4) short lifespan (approximately 18 months); and (5) estrogen biology similar to humans. In addition, sexual

dimorphism, based on the morphology of the anal fin (Figs 1.6 – 1.7), is very prominent in *O. melastigma* ca. 1-M after hatching, rendering it highly desirable for gender study.

Fig 1.6 The marine medaka *Oryzias melastigma*. Male's anal fin.

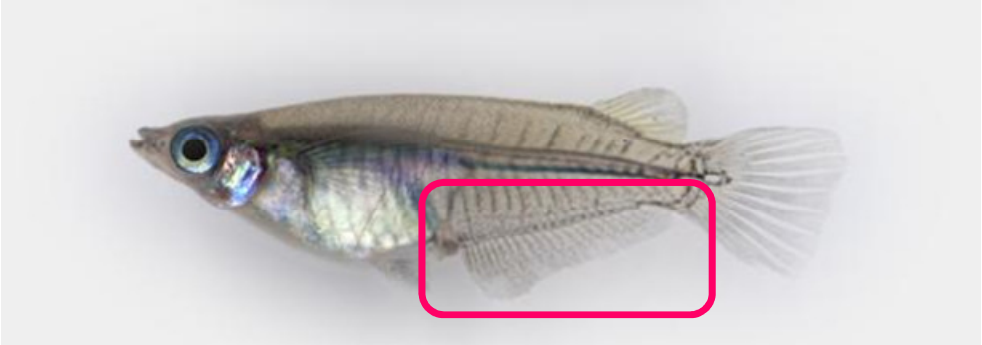


Fig 1.7 The marine medaka *Oryzias melastigma*. Female's anal fin.

Furthermore, our group has already conducted various aspects of research on telomere and telomerase in *O. melastigma* (Yu et al. 2006; Kong et al. 2008; Mok 2008; Chen 2011). Mok (2008) has optimized a cost-effective and high throughout real-time quantitative telomeric repeat amplification protocol (RTQ-TRAP) assay for

the quantification of telomerase activity in fish, including *O. melastigma*. We have also successfully developed protocols to localize and quantify the TERT gene through *in situ* hybridization (ISH) and the TERT protein through immunohistochemistry (IHC) in multiple tissues within a single adult medaka fish (Kong et al. 2008; Chen 2011). These platforms support the application of *O. melastigma* for studying the *in vivo* mechanistic relationship between estrogen, telomere dynamics and telomerase.

1.9 Research hypothesis, aim and objectives of this study

It has been well established in mammalian models that TL and E2 are involved in aging; however, these relationships have not been validated and remain largely unknown in non-mammalian models. Whether TL and E2 are an explanation for LGG is still poorly understood especially in non-mammals. The present study explores the relationship between E2, TL and aging using *O. melastigma* as an alternative model. Given the evidence for the role of TL and E2 on aging in mammalian models, it is hypothesized that a good inverse relationship exists between E2 and TL in aging, and that E2 and TL are potential causes for LGG in *O. melastigma*.

The specific objectives of the present study are to:

1. determine if LGG (females living longer than males) exists in *O. melastigma*;

2. study the effect of gender on age-dependent changes in plasma E2, T and E2/T ratio;
3. study the effect of gender on age- and tissue-dependent changes in TL;
4. study the effect of gender on age- and tissue-dependent changes in TERT, ERα expression and telomerase activity;
5. study the effect of gender on age- and tissue-dependent changes in DNA and protein oxidation

Results of this study would provide a comprehensive understanding of the relationship between E2, TL and aging in fish. If good correlations exist between E2, TL and aging in *O. melastigma*, this may shed light on the possible mechanisms of gender-specific longevity in fish.

CHAPTER 2 MATERIALS AND METHODS

2.1 Materials

Unless otherwise specified, all reagents, chemicals and solvents used in this study were of analytical and/or molecular biology grade.

2.2 Sex ratio and fish survival

The marine medaka *Oryzias melastigma* were obtained from the (then) Center of Coastal Pollution and Conservation, CCPC (now the State Key Laboratory in Marine Pollution), City University of Hong Kong. Aquarium fish were maintained under optimal growth and breeding conditions in 30‰ artificial seawater, filtered and oxygenated water at 28 ± 2^{o}C under a 14:10 h light:dark cycle. An average of 250 marine medaka of the same age (± 2 weeks) were maintained in a 500-L glass fish tank. The sex ratio of *O. melastigma* was determined for age group at 4-months, 8-months and 12-months (n = 3 replicate tanks). Female and male *O. melastigma* at 4-months, 8-months and 12-months old are shown in Figs 2.1 – 2.3. In a separate tank of 8-month old *O. melastigma* (containing 98 females and 101 males) mortality was checked and recorded daily over 16 weeks to determine fish survival over time.

Fig 2.1 Marine medaka *Oryzias melastigma* at 4-months old: male (top) and female (bottom).

Fig 2.2 Marine medaka *Oryzias melastigma* at 8-months old: male (top) and female (bottom).

Fig 2.3 Marine medaka *Oryzias melastigma* at 12-months old: male (top) and female (bottom).

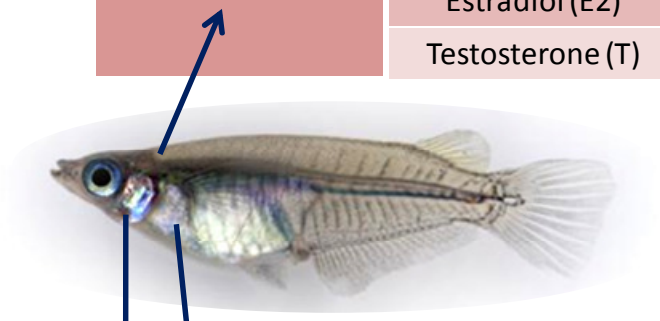
2.3 Age and gender specific changes in *O. melastigma*

O. melastigma at 4-months, 8-months and 12-months old (n = 90, 45 females and 45 males for each age group) were sampled for multiple analyses (Fig. 2.4). At 4-months old, the young *O. melastigma* were sexually mature, the amount of genomic DNA in their organs (liver and gills) was sufficient to perform DNA analyses on a reasonable pool of fish (> 3 μg for each sample pooled from 9 fish) (See Sections 2.6 and 2.9). At 8-months old, the adult *O. melastigma* was at the peak of reproductive capability, as indicated by the highest amount of egg production (unpublished data of Prof. Rudolf Wu's group). At 12-months old, the aged *O. melastigma* showed declining reproductive capability and an increasing mortality rate (Fig 3.1).

The liver and gill were chosen for the study because preliminary study of my teammate (Ms. Vicky Y.Y. Wong, City University of Hong Kong) showed that significant gender difference in mean TL was detected in these two organs, but not in brain and intestine of 4-months old *O. melastigma*. Besides, sufficient genomic DNA (> 3 μg) could be extracted on a reasonable pool of nine small sized 4-months old *O. melastimga*, whereas a pool of > 30 individuals of 4-months fish was needed for the heart and muscle.

Fish were first anesthetized by chilling in ice water. The body length and body weight of each fish were measured. The anesthetized fish was sacrificed by cutting the

spinal cord, blood was collected from the aorta near the spine (Section 2.4), and the gills and liver were isolated (Section 2.5).



Blood	End points	Measurement method
	Estradiol (E2)	ELISA (E2)
	Testosterone (T)	ELISA (T)

Gills / Liver	End Points	Measurement method
	Telomere length	Southern blotting
	Telomerase activity	RTQ-TRAP
	TERT expression	Real-time RT-PCR
	ERα expression	Real-time RT-PCR
	DNA oxidation	ELISA (8-OHdG)
	Protein oxidation	ELISA (Protein carbonyl)

Fig 2.4 A schematic diagram showing the overall experimental plan.

2.4 Blood collection and hormones analyses

A 2 µl of whole blood was collected from each fish. Blood collected from 3 fish was pooled as one replicate (6 µl) and diluted to 500 µl by adding 494 µl of sterile deionized water (Milli Q_{PLUS}). 2 ml of diethyl ether was added to the diluted blood for

extraction of sex steroid hormones. The mixture was vortexed and then centrifuged at 3,000 rpm at room temperature for 10 minutes. The diethyl ether (the upper organic layer) was transferred to a clean glass tube using a clean glass Pasteur pipette. The diethyl ether extraction procedure was repeated two more times. The diethyl ether collected was completely evaporated with a gentle stream of dry nitrogen, and the remaining extracts were kept at -80^{o}C for sex hormones analyses (n = 15 replicates for each age group and each gender).

The levels of plasma estradiol and testosterone in *O. melastigma* were measured by Enzyme-Linked ImmunoSorbent Assay (ELISA) using the Estradiol EIA kit (Cayman Chemical Company) and the Testosterone EIA kit (Cayman Chemical Company), respectively, according to the manufacturer's instructions.

2.5 Organ isolation

Immediately after blood collection, the gills and liver were carefully dissected from the fish (kept under ice-cold phosphate-buffered saline). Each isolated organ was cut into three equal portions. The ⅓ liver/gill portion from each of the same three fish used for blood sampling (Section 2.4) were pooled as one replicate for RNA extraction (for TERT and ERα mRNA expression), the other ⅓ liver/gill portion were pooled for protein extraction (for telomerase activity and protein oxidation) (n=15 for

each age group and each gender). For DNA extraction (for telomere length and DNA oxidation), the remaining ⅓ liver/gill portion were pooled from 9 fish as one replicate to obtain a sufficient amount of genomic DNA to perform Southern blotting (> 3 μg for each sample was needed) (n = 5 for each age group and each gender). Correlation analyses among different end-points became feasible for the same fish sample groups. The pooled organs were snap-frozen in liquid nitrogen and kept at -80^{o}C for further analysis.

2.6 Telomere length measurement by Southern blotting

Southern blotting is a technique developed for detection and quantification of nucleic acid for specific DNA or RNA sequence within a mixture (Southern 2006). Genomic DNA is first digested with restriction enzymes into DNA fragments (see 2.6.3). The fragments are then separated by gel electrophoresis. The DNA fragments on gel are transferred to a nylon membrane by capillary action. The DNA fragments with specific sequence on the membrane can be detected by nucleic acid hybridization using labeled probes. For a detail protocol, see also Southern (2006).

2.6.1 Genomic DNA extraction

Genomic DNA of pooled livers/gills was extracted using DNeasy® Blood and

Tissue kit (Qiagen) according to the manufacturer's instructions.

2.6.2 DNA precipitation

A 400 µl of extracted genomic DNA was mixed with 40 µl of 3M sodium acetate (pH 5.2) and 1 ml of ice-cold absolute ethanol. The mixture was incubated at -20^{o}C for 2 hours, and then centrifuged at 14,000 rpm for 15 minutes at 4^{o}C. The supernatant was discarded and 1 ml of 70% ethanol was subsequently added to re-suspend the DNA. The mixture was centrifuged again at 14,000 rpm for 15 minutes at 4^{o}C, and the supernatant was discarded. The DNA pellet was air-dried for 5 minutes before being dissolved in 40 µl of nuclease-free water at 65^{o}C for 10 minutes (the DNA extract).

2.6.3 Restriction enzyme digestion

The DNA extract was digested with HinfI and RsaI (New England BioLabs) at 37^{o}C overnight so that the restriction enzymes could cut the double-stranded DNA at the restriction sites. Table 2.1 shows the restriction sites of HinfI and RsaI, respectively. A 2 µl of digested DNA was electrophoresized in 1% agarose gel to ascertain complete digestion.

Table 2.1 Restriction sites of HinfI and RsaI. The restriction sites were obtained from New England BioLabs (www.neb.com).

Restriction enzyme	Restriction site
HinfI	5′...G▼ANTC...3′ 3′...CTNA▲G...5′
RsaI	5′...GT▼AC...3′ 3′...CA▲TG...5′

2.6.4 Probe preparation

To detect the DNA fragments with telomere sequence (TTAGGG repeats), DIG-labelled $TTAGGG_5$ oligonucleotide probes were prepared. $TTAGGG_5$ oligonucleotide probes (Invitrogen) were DIG-labeled using DIG Oligonucleotide 3'-End Labeling Kit 2nd Generation (Roche Applied Science) according to the manufacturer's instructions.

2.6.5 Electrophoresis and blotting

A 3 µg of completely digested genomic DNA was electrophoresized in 1% agarose electrophoresis in 1x Tris-acetate-EDTA (TAE) buffer (40 mM Tris acetate, 1 mM EDTA, pH 8.0) with 10% of 0.5 µg/ml ethidium bromide at 70 – 80 V for 6 – 8 hours. This was run in parallel with 10 µl of 0.1 µg/µl of GeneRuler™ DNA Ladder

Mix (Fermentas) and 2 µl of 0.5 µg/µl of Lambda DNA / EcoRI + HindIII (Fermentas) as the DNA molecular weight markers. The DNA was then de-purinated by soaking the gel in 250 mM HCl solution for 5 minutes, de-naturation solution (0.5 M NaOH and 1.5 M NaCl) for 30 minutes and neutralization solution (0.5 M Tris-HCl, pH 7.2, 1.5 M NaCl and 100 mM EDTA) for 30 minutes. The DNA on the gel was then blotted onto a positively charged nylon membrane (Hybond XL, Amersham) by capillary transfer using 10x saline-sodium citrate (SSC) buffer (1.5 M NaCl, 150 mM sodium citrate, pH 7.0). After overnight DNA transfer, the nylon membranes were UV-crosslinked by exposure to 150 mJ UV-C for 150 seconds in a GS Gene Linker UV Chamber (Bio-Rad).

2.6.6 Hybridization and detection

DNA blotted nylon membranes were rinsed with 10x SSC, and pre-hybridized with ExpressHyb solution (Clontech) at 42^{o}C for 30 minutes inside a hybridization incubator (Hybridization incubator combi-H12, FINEPCR). A 10 µl of DIG-labeled $TTAGGG_5$ oligonucleotide probes were de-natured at 95^{o}C for 5 minutes using PTC-200 Peltier Thermal Cycler (Bio-Rad), quenched on ice for 1 minute and added to 10 – 15 ml ExpressHyb solution in which the DNA blotted nylon membranes were incubated overnight at 42^{o}C. After hybridization, the membranes were washed twice

for 5 minutes in 2x SSC with 0.1% SDS at 25°C and twice for 15 minutes in 0.1x SSC with 0.1% SDS at 42°C. The membranes were further washed in washing buffer (0.1M NaCl, 0.15M maleic acid, pH 7.5 and 0.3% Tween 20) at room temperature for 5 minutes before incubation in blocking solution (0.1M NaCl, 0.15M maleic acid, pH 7.5 and 1% w/v blocking reagent from Roche Applied Science) for 30 minutes and then in anti-DIG-AP conjugate (Roche, 1:10,000 in blocking solution) for 2 – 4 hours. Finally, the membranes were incubated for 5 minutes in detection buffer (0.1M Tris-HCl, pH 9.5 and 0.1M NaCl) and a 10 ml of CDP-star (Roche, 1:200 in the detection buffer) was subsequently added to the membranes according to the manufacturer's instructions. The membrane was then exposed to Hyperfilm™ ECL (Amersham) in the dark for 5 minutes.

2.6.7 Computerized telomere analysis

The exposed films were scanned using Duoscan HiD scanner (AGFA) with software Agfa FotoLook 3.60.00 and saved as TIFF files. The files were analyzed using ImageJ (NIH, freely available at rsbweb.nih.gov/ij/) to determine the distribution of length of the telomere restriction fragments (TRFs) in each sample. Quantification of the proportion of TRFs greater than 5 kb, 5 – 4 kb, 4 – 3 kb, 3 – 2 kb and less than 2 kb was carried out using TeloRun (freely available at

www4.utsouthwestern.edu/cellbio/shay-wright/research/1UTSWTelorunforweb.xls) using the equation as follows:

$$\text{Proportion of TRFs} = \sum(OD_i)/ \sum(OD_{total})$$

where OD_i refers to the optical density above background within interval i (< 5 kb, 5 – 4 kb, 4 – 3 kb, 3 – 2 kb or > 2 kb) and OD_{total} refers to the total optical density above background.

2.7 Telomerase activity measurement by Real-Time Quantitative Telomeric Repeat Amplification Protocol (RTQ-TRAP) assay

The Real-Time Quantitative Telomeric Repeat Amplification Protocol (RTQ-TRAP) assay was developed (Hou et al. 2001; Wege et al. 2003) to quantify telomerase activity using real-time polymerase chain reaction (PCR) technique. Firstly, oligonucleotide telomerase substrate primers were elongated by telomerase enzyme present in tissue lysate. The extended telomeric products were then amplified exponentially by PCR. Elongation of the telomerase substrate primer was determined by the fluorescence signal generated by SYBR Green, a fluorescent dye which can emit fluorescent signal when it binds to double-stranded PCR products. The RTQ-TRAP assay used in the present study followed the protocol optimized by Mok (2008) for fish, including *O. melastigma*. (For details of the procedures, see also Mok

2008).

2.7.1 Protein extraction

Proteins of the pooled livers/gills sample were extracted with 250 µl CHAPS (3-[(3-Cholamidopropyl)-dimethyl-ammonio]-1-propane sulfonate)-containing lysis buffer [10 mM Tris-HCl, pH 7.5, 1 mM $MgCl_2$, 1 mM EGTA, pH 8.0, 10% glycerol, 0.5% (v/v) CHAPS, 0.1 mM PMSF (phenylmethylsulfonyl fluoride), 5 mM beta-mercaptoethanol] with the aid of a polypropylene pellet pestle. The extract was incubated on ice for 30 minutes before centrifugation at 14,000 rpm at 4^{o}C for 30 minutes. The supernatant (protein lysate) was collected, snap-frozen in liquid nitrogen and stored at -80^{o}C for further analysis.

2.7.2 Determination of protein concentration

The protein concentration in tissue lysate was measured with Bio-Rad Protein Assay. Bovine serum albumin (BSA) (Sigma-Aldrich) was used as the protein standard. The BSA (1.96 mg, Sigma-Aldrich) was re-constituted with 4.9 ml distilled water (Invitrogen) to make up a 400 µg/ml solution which was then serially diluted with distilled water (Invitrogen) to get standard concentrations of 200 µg/ml, 100 µg/ml, 50 µg/ml and 25 µg/ml. Tissue lysate was diluted 30 times with CHAPS lysis buffer so that the absorbance fell within the BSA standard curve. The protein assay

buffer was diluted 5 times with MilliQ water and then filtered through a 0.45 μm syringe filter to remove any particulates. A 10 μl of diluted protein standard or lysate was aliquoted into each well of a 96-well plate (Greider), followed by the addition of 200 μl of diluted Bio-Rad protein assay buffer. The reaction mixture was allowed to incubate at room temperature for 5 minutes, and scanned at a wavelength of 595 nm using a spectrophotometer (Spectra MAX 340, Molecular Devices). The absorbance data were analyzed using the SOFTmax Pro 3.1.1 software to calculate protein concentration in each tissue lysate. Protein concentration in the lysate was determined from the BSA standard curve and multiplied by the dilution factor. Each sample was analyzed in triplicate to ensure reproducibility.

2.7.3 RTQ-TRAP assay

RTQ-TRAP assay was performed using the ABI 7500 Fast Real-Time PCR System (Applied Biosystems), with each reaction mixture containing 20 μl of SYBR Green Buffer [20 mM Tris-HCl, pH 8.3, 63 mM KCl, 3.5 mM $MgCl_2$, 1 mM EGTA, pH 8.0, 0.1 mg/ml BSA, 0.005% Tween 20, 100 μM dNTPs, SYBR Green I (1:25,000), 10 nM ROX^{TM} reporter dye, 1.25 U HotStar Taq Polymerase (Qiagen)], 40 ng protein lysate (see Section 2.7.1), 0.1 μg telomerase substrate (TS) primer (HPLC grade, Proligo) and 0.065 μg anchored return (ACX) primer (HPLC grade,

Proligo). The sequences of TS and ACX primers are listed in Table 2.2. The reaction began with incubation at 25°C for 30 minutes for TS primer extension by the enzyme telomerase, followed by incubation at 95°C for 15 minutes to inactivate telomerase and to activate the HotStar Polymerase. The reaction was immediately subject to 40 cycles of 95°C for 30 seconds, 60°C for 30 seconds and 72°C for 1 minute. Melting curve analysis was automatically carried out after the fortieth cycle to verify the amplification specificity. Lysate-free control (containing SYBR Green buffer, TS and ACX primers alone) was included as the no-template control (NTC). Each sample was run in triplicate to ensure reproducibility. The C_t values were determined from semi-log amplification plots (log increase in fluorescence signal against cycle number). The relative telomerase activity was calculated by the $2^{-\text{delta Ct}}$ method (Livak and Schmittgen, 2001) according to the equation:

$$\text{Relative TA} = 2^{-(\text{Ct of fish 1} - \text{Ct of fish 2})}$$

The telomerase activity of the first control fish was set as 1 and the rest of the samples were calculated with reference to the selected control.

Table 2.2 Sequences of TS and ACX primers (Hou et al. 2001).

Primer name	Sequence
TS	5'-AATCCGTCGAGCAGAGTT-3'
ACX	5'-GCGCGG$(CTTACC)_3$CTAACC-3'

2.8 Q-PCR measurement of TERT and ERα mRNA expressions

2.8.1 RNA extraction

The total RNA of the pooled livers/gills was extracted using Trizol reagent (Invitrogen) according to the manufacturer's instructions. In brief, fish tissue in a 1.7 ml microcentrifuge tube was homogenized at 19,000 rpm (Ystral® homogenizer D-79282) for 1 minute in 1 ml ice-cold Trizol reagent until no visible tissue clumps remained. The homogenized sample was incubated at room temperature for 5 minutes. A 200 µl of chloroform was added and the mixture was shaken vigorously for 15 seconds before incubation at room temperature for 3 minutes. After centrifugation at 12,000 x g for 15 minutes at 4°C, the colorless upper aqueous layer was transferred to a clean 1.7 ml micro-centrifuge tube. A 500 µl of isopropanol was added and the tube was inverted several times to mix the contents completely. After resting at room temperature for 10 minutes, the RNA in the mixture was precipitated by centrifugation at 12,000 xg for 10 minutes at 4°C. The RNA pellet was washed with 1

ml ice-cold 75% ethanol and centrifuged again at 12,000 xg for 10 minutes at 4°C. The ethanol solution was discarded and the RNA pellet was air-dried for 10 minutes. The RNA pellets of gill and liver samples were re-suspended with 20 µl and 60 µl Invitrogen DNase- and RNase-free distilled water, respectively, since RNA in the liver is usually more concentrated than that in the gill. The concentration and purity of RNA was measured by absorbance at 260 nm and 280 nm, respectively, using a BioPhotometer (Eppendorf). The RNA quality was determined by 1% agarose gel electrophoresis. The purified RNA pellets were stored at -80°C for further analysis.

2.8.2 Reverse transcription

Prior to reverse transcription, the purified RNA was treated with RQ1 RNase-free DNase (Promega) to digest the DNA. First, 1 µg of RNA was dissolved in 8 µl of RNase-free water. Then, 1 µl of 10 x Reaction buffer (Promega, provided together with RQ1 RNase-free DNase kit) and 1 µl of DNase (1 U/µl, Promega) were added. The mixture was incubated at 37°C in a Peltier Thermal Cycler (PTC-200, MJ Research) for 30 minutes. The Stop solution (Promega, provided together with RQ1 RNase-free DNase kit) was subsequently added into the mixture and incubated at 65°C for 10 minutes to inactivate the DNase. After DNase digestion, the first strand complementary DNA (cDNA) was synthesized using M-MLV Reverse Transcriptase,

RNase H Minus, Point Mutant (Promega). A 3 μl of random primer (40 ng/μl) was added to the mixture which was heated to 70°C for 5 minutes and immediately cooled on ice for 5 minutes. This was followed by incubation with the RT master mix [containing 1 x M-MLV reverse transcriptase reaction buffer, 500 μM dNTPs, 40 units RNase OUT (Invitrogen) and 200 units M-MLV reverse transcriptase (H^-)] at room temperature for 10 minutes and then at 42°C for 50 minutes. Finally, the reaction was stopped by heating at 70°C for 15 minutes. The cDNA was stored at -20°C until use.

2.8.3 Real-time Polymerase Chain Reaction (PCR)

The expressions of omTERT and omERα mRNA were determined by SYBR Green I-based real-time PCR in an ABI 7500 Fast Real-time PCR System (Applied Biosystems). The omTERT-, omERα- and reference gene om18S-pecific primers were kindly provided by Ms. Helen O.L. Mok, City University of Hong Kong. The sequences of these primers are listed in Table 2.3. The first strand cDNA was diluted 10 times with nuclease-free water. The PCR was carried out in a final volume of 25 μl [composed of 5 μl of diluted cDNA template, 1 x Power SYBR® Green PCR Master Mix (Applied Biosystems) and 400 ng each of the forward and reverse primers]. omTERT gene expression was measured with primers omTERT-real-F and

omTERT-real-R. omERα gene expression was measured with primers omERα-real-F and omERα-real-R. The PCR was started by incubation at 95°C for 10 minutes, followed by 40 cycles at 95°C for 15 seconds and at 60°C for 1 minute. Melting curve analysis was subsequently performed to verify the amplification specificity.

Table 2.3 Gene-specific primers used for quantification of omTERT, omERα and om18S rRNA.

Primer name	Sequence
omTERT-real-F	5'-CTGATGTGGAAGATGAAGGTGA-3'
omTERT-real-R	5'-AGAAGCCAGGTCAGAAACAGAC-3'
omERα-real-F	5'-CGCTTCCGTGTGCTCAAACTC-3'
omERα-real-R	5'-GTATCCAGCATGCTCTGAACC-3'
om18S-real-F	5'-GACAAATCGCTCCACCAACT-3'
om18S-real-R	5'-CCTGCGGCTTAATTTGACCC-3'

Normalization of cDNA loading was performed by the determination of the amount of 18S rRNA in the corresponding samples. The first strand cDNA was diluted 5,000 times with nuclease-free water. A 5 μl of diluted cDNA was mixed with the SYBR Green master mix, 400 ng om18S-real-F and om18S-real-R primers. The

thermal cycle for om18S was the same as the procedures described for the determination of omTERT and omERα expressions.

2.8.4 Quantification of relative gene expression

The expression of a target gene was normalized to 18S rRNA, resulting in a mean normalized expression (MNE) value (Simon, 2003), according to the equation below:

$$MNE = (E_{18S\ rRNA})^{Ct\ of\ 18S\ rRNA} / (E_{target\ gene})^{Ct\ of\ target\ gene}$$

where $E = 10^{-1/slope}$ represents primer efficiency which is determined by standard curve (Simon, 2003). The primer efficiencies were listed in Table 2.4 (provided by Ms. Helen O.L. Mok, City University of Hong Kong). Determination of the fold differences between samples was based on the equation as follows:

$$Fold\ change = MNE_{sample}/MNE_{control}$$

The gene expression of the first control fish was set as 1 and the rest of the samples were calculated with reference to the selected control.

Table 2.4 Primer efficiencies of the 3 primer pairs used to measure omTERT, omERα and om18S gene expression.

Primer pair	Primer efficiency (E)
omTERT-real-F x omTERT-real-R	98.6%
omERα-real-F x omERα-real-R	100.0%
om18S-real-F x om18S-real-R	96.7%

2.9 DNA and protein oxidation measurement by ELISA

The level of DNA oxidation, as indicated by 8-OHdG level, was measured for each genomic DNA extract (see Section 2.6.1) using the 8-hydroxy-2-deoxy Guanosine EIA kit (Cayman Chemical Company) according to the manufacturer's instructions.

The level of protein oxidation, as indicated by protein carbonyl level, was measured for each tissue protein lysate (see Section 2.7.1) using the Protein Carbonyl Assay kit (Cayman Chemical Company), according to the manufacturer's instructions.

2.10 Statistical analysis

Statistical analyses were performed using software SigmaStat 3.0.1 (SPSS) with graphs plotted using software GraphPad Prism 2 (GraphPad Software, Inc.). Data was first tested for normality and equal variance using the Kolmogorov-Smirnov test with Lilliefors' correction and the equal variance test, respectively. If the data passed both the tests, unpaired t-tests were performed to test the null hypothesis that there was no significant gender difference in body length/body weight/plasma E2 level/plasma T level/plasma E2 to T ratio as well as the proportions of telomere restriction fragments (TRFs) (from < 2 kb to > 5 kb in length)/telomerase activity/mRNA

expressions/8-OHdG level/protein carbonyl level in liver/gills; otherwise Mann-Whitney Rank Sum Tests were performed. Differences were regarded as significant if $p \leq 0.05$.

Kruskal-Wallis One-Way Analysis of Variance (ANOVA) was used to test the null hypothesis that there was no significant change in body length/body weight/plasma E2 level/plasma T level/plasma E2 to T ratio, as well as the proportions of telomere restriction fragments (TRFs) (from < 2 kb to > 5 kb in length)/telomerase activity/mRNA expressions/8-OHdG level/protein carbonyl level over age (4-months, 8-months, 12-months) in liver/gills. If a significant difference ($p \leq 0.05$) was detected, pairwise comparison was performed using Dunn's method.

Spearman rank order correlation was performed to investigate if a significant correlation was present (1) between plasma E2 level and telomerase activity/TERT and ERα mRNA expression in liver/gills, (2) between telomerase activity, TERT and ERα mRNA expression in liver/gills, and (3) between plasma E2 and 8-OHdG/protein carbonyl levels in liver/gills.

CHAPTER 3 RESULTS

3.1 Sex ratio

The sex ratio [male:female] of marine medaka *Oryzias melastigma* at 4 months and 8 months old was averaged at 1.03 and 1.15, respectively, which was very close to 1:1 (Tables 3.1). At 12 months old, the sex ratio of marine medaka dropped to 0.63, i.e. ca. 2:3 for males:females (Table 3.1).

Table 3.1 Sex ratios of marine medaka *Oryzias melastigma* from 4 months to 12 months old.

Stock Culture	Female	Male	Sex ratio [Male(s)/Female]
		4 months	
Tank 1.1	162	174	**1.07**
Tank 1.2	186	176	**0.95**
Tank 1.3	212	227	**1.07**
Total	560	577	**1.03**
		8 months	
Tank 2.1	74	90	**1.22**
Tank 2.2	98	101	**1.03**
Tank 2.3	147	176	**1.20**
Total	319	367	**1.15**
		12 months	
Tank 3.1	45	15	**0.33**
Tank 3.2	55	45	**0.82**
Tank 3.3	70	47	**0.67**
Total	170	107	**0.63**

3.2 Fish survival over time

The mortality of female (n = 98) and male (n = 101) *O. melastigma* at 8 months old was checked and recorded daily over 16 weeks (equivalent to 12 months old). After 8 weeks, i.e. at the age of 10 months old onwards, the number of surviving female medaka started to outweigh their male counterparts (Fig 3.1).

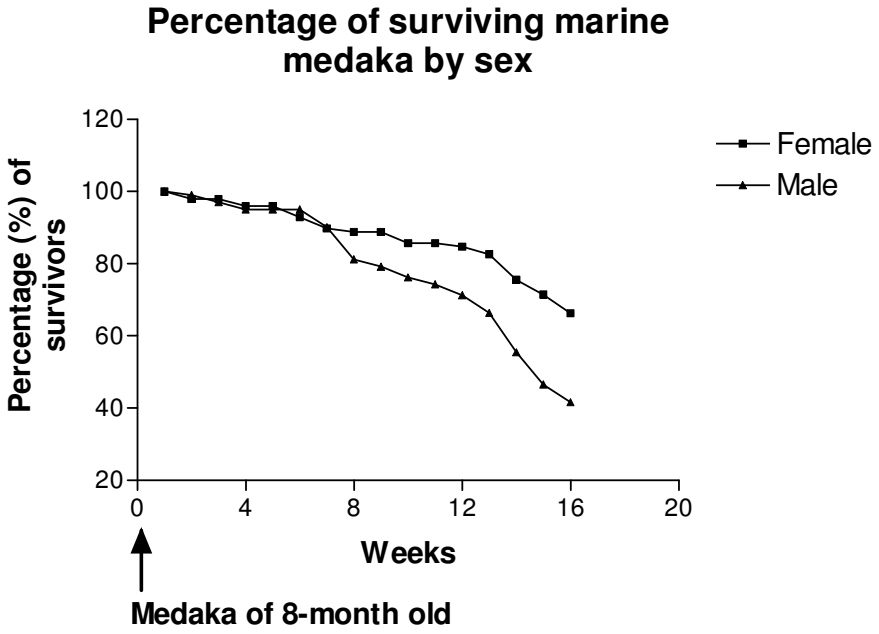


Fig 3.1 Percentage of surviving female and male *Oryzias melastigma* over time after 8 months old.

3.3 Body length and body weight

No significant gender difference was detected in the body length of *O. melastigma* at 4 months, 8 months and 12 months old (Fig 3.2a). The body weight of the female *O. melastigma* was generally higher than that of the male, and significant difference was observed between the genders (female > male) at 4 months and 12 months old (Fig 3.2b). Overall, the body weight of 8-month and 12-month old medaka was almost double that of the 4-month old fish (Kruskal-Wallis One-Way ANOVA, $p \leq 0.001$) (Fig. 3.2b).

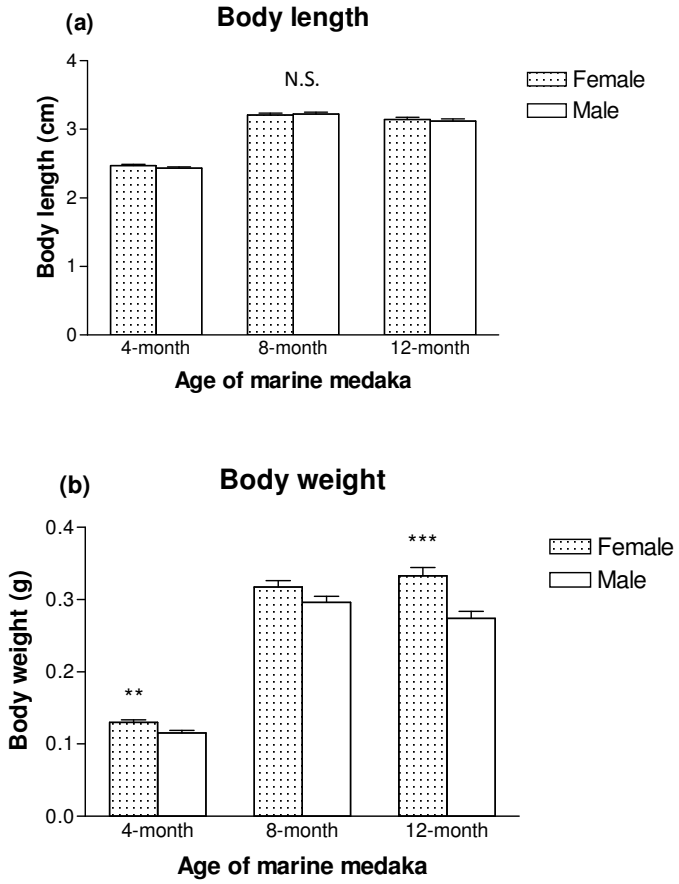


Fig 3.2 Body length (a) and body weight (b) of female and male marine medaka Oryzias melastigma at 4 months, 8 months and 12 months old. Data are expressed as mean ± standard error of mean (n = 44 – 45). Significant differences between the sexes are indicated by asterisks (**, $p \leq 0.01$; ***, $p \leq 0.001$). N.S. = no significant difference.

3.4 Plasma E2 level, T level and E2 to T ratio

Plasma E2 level was significantly higher in females than males at 4 months ($p \leq 0.001$) and 8 months old ($p \leq 0.001$) (Fig 3.3a). Likewise, a higher plasma T level in males than females was found at 4 months ($p \leq 0.05$) and 8 months old ($p \leq 0.001$) (Fig 3.3b). At 12 months old, no significant gender difference in both plasma E2 and

T levels was detected in *O. melastigma* (Fig 3.3a, b), which was due mainly to a decline of plasma E2 level in females and a drop of plasma T level in males (Kruskal-Wallis One-Way ANOVA, $p \leq 0.001$) (Fig 3.3a, b).

The plasma E2 to T ratio in females was higher than males at 4 months ($p \leq 0.001$) and 8 months old ($p \leq 0.001$), whereas no significant difference between gender was found in marine medaka of 12 months old (Fig 3.3c). In both females and males, plasma E2 to T ratio peaked at 4 months (Kruskal-Wallis One-Way ANOVA, $p \leq 0.001$) (Fig 3.3c).

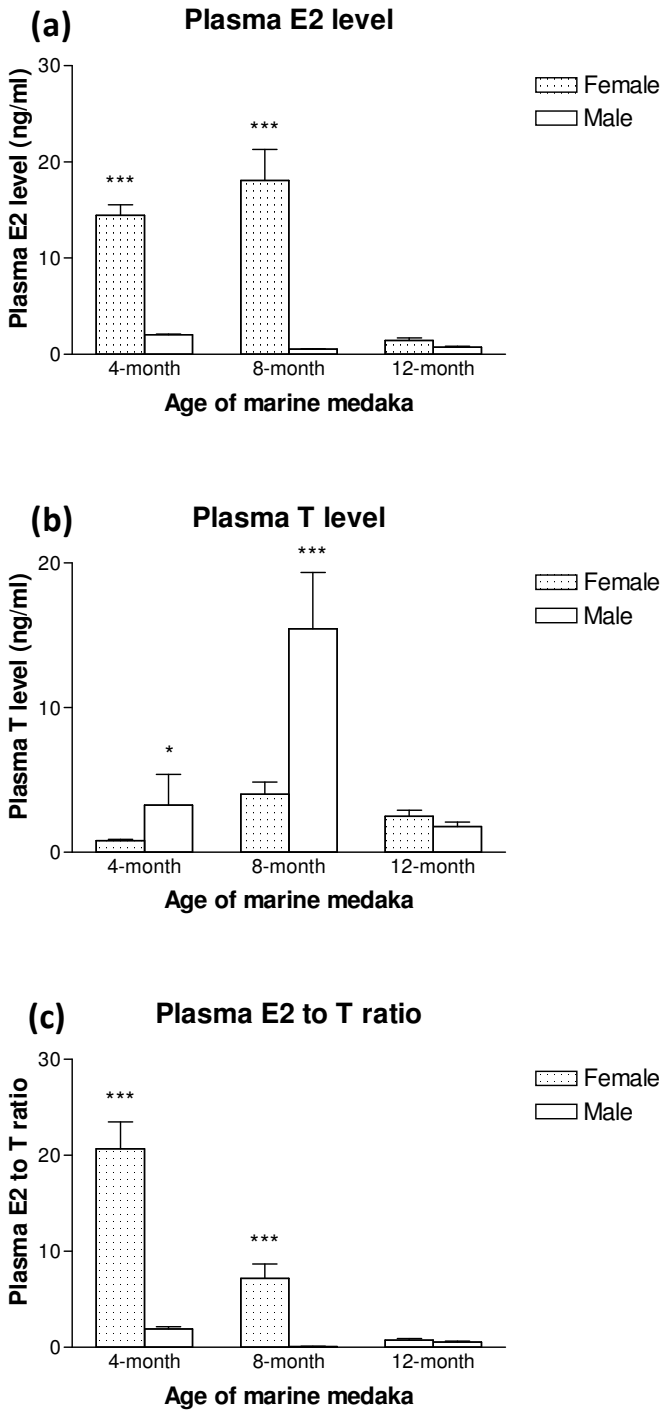


Fig 3.3 Age and gender difference in plasma E2 (a), plasma T (b) and plasma E2 to T ratio (c) in marine medaka *Oryzias melastigma* at 4 months, 8 months and 12 months old. Data are expressed as mean ± standard error of mean (n = 15). Significant differences between the sexes are indicated by asterisks (*, $p \leq 0.05$; ***, $p \leq 0.001$).

3.5 Telomere length in liver

A significant increase in the proportion of short telomere restriction fragments (TRFs < 2 kb) was found in the liver of both female and male *O. melastigma* with advancing age ($p \leq 0.01$) (Fig 3.4). Consistent with this, a reduction in the proportion of long TRFs (> 5 kb) was also found in the liver of *O. melastigma* with age, and a significant reduction was detected in aging females ($p \leq 0.01$) (Fig 3.4).

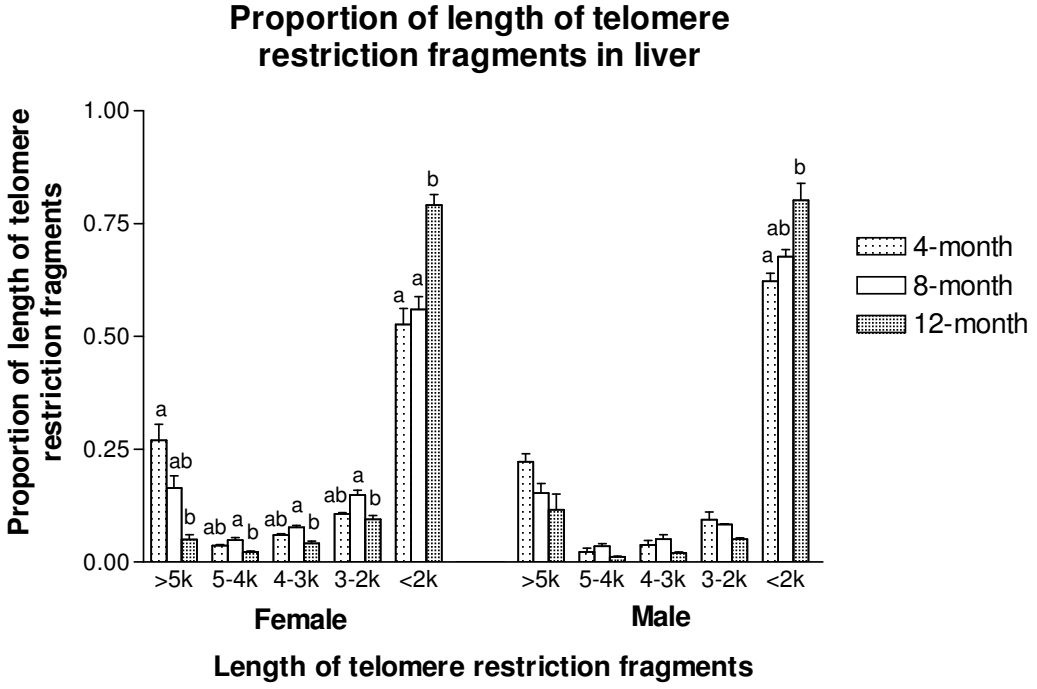


Fig 3.4 Change in the proportion of telomere restriction fragments (TRF from < 2 kb to > 5 kb in length) in the liver of female and male marine medaka *Oryzias melastigma* from 4 months to 12 months old. Data are expressed as mean ± standard error of mean (n = 5). Different letters represent significant differences by age ($p \leq 0.05$).

Fig 3.5 illustrates gender difference in liver TRFs with age. At 4 months old, the proportion of short TRFs (< 2 kb) in the females was significantly lower than that of the males ($p \leq 0.05$), while no significant gender difference was found in other TRF proportions (Fig 3.5). At 8 months, the proportion of short TRFs (< 2 kb) remained significantly lower in the liver of the females compared to that of the males ($p \leq 0.01$), while the proportions of TRFs between 2 – 3 kb ($p \leq 0.01$) and between 3 – 4 kb ($p \leq 0.05$) were higher in the females than the males (Fig 3.5). At 12 months old, the proportion of short TRFs (< 2 kb) was similar between the males and females, while higher proportions of TRFs between 2 – 3 kb ($p \leq 0.01$), 3 – 4 kb ($p \leq 0.05$) and 4 – 5 kb ($p \leq 0.05$) were found in the females than that of the males (Fig 3.5).

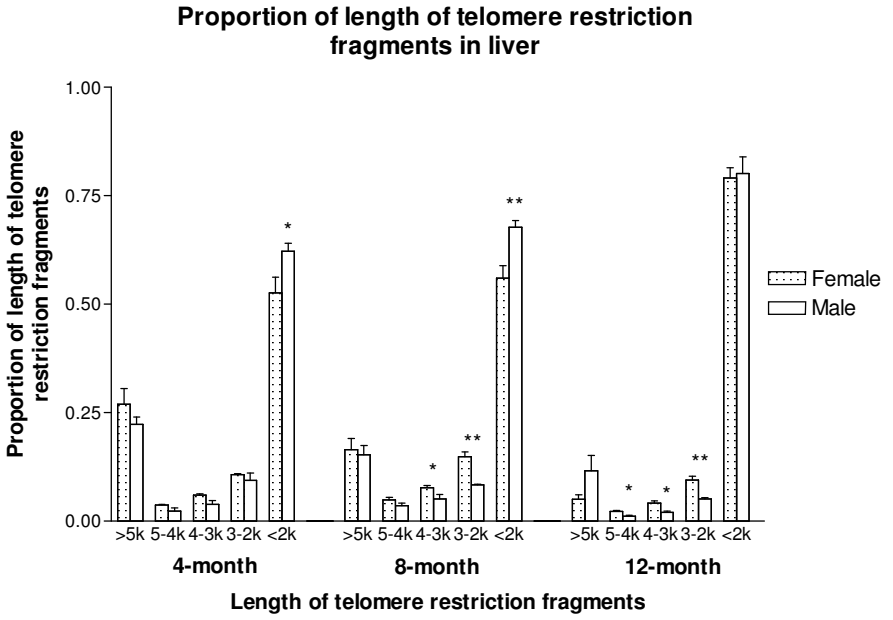


Fig 3.5 Change in proportion of telomere restriction fragments (TRF from < 2 kb to > 5 kb in length) in the liver of male and female *Oryzias melastigma* at different ages (4 to 12 months old). Data are expressed as mean ± standard error of mean (n = 5). Significant differences between the sexes are indicated by asterisks (*, $p \leq 0.05$; **, $p \leq 0.01$).

3.6 Telomere length in gill

In the females, the proportion of short TRFs (< 2 kb) was significantly higher in the gills of *O. melastigma* with advancing age ($p \leq 0.05$) (Fig 3.6), which was concomitant with a reduction in proportions of medium size TRFs ($p \leq 0.05$) (Fig 3.6). In the males, no significant difference was found, with age, in any proportions of TRFs in the gills of *O. melastigma* (Fig 3.6).

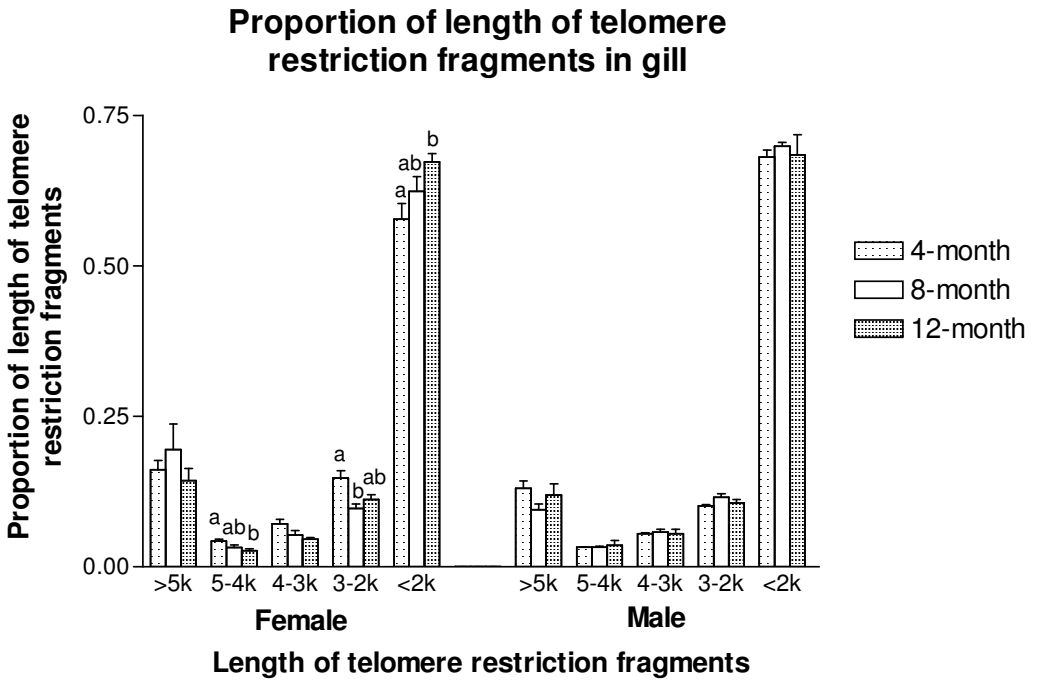


Fig 3.6 Change in proportion of telomere restriction fragments (TRF from < 2 kb to > 5 kb in length) in the gills of female and male marine medaka *Oryzias melastigma* from 4 months to 12 months old. Data are expressed as mean ± standard error of mean (n = 5). Different letters represent significant differences by age ($p \leq 0.05$).

Fig 3.6 illustrates gender difference in gill TRFs with age. At 4 months old, female *O. melastigma* exhibited a lower proportion of short TRFs (< 2 kb) ($p \leq 0.01$), but higher proportions of medium TRFs between 2 – 3 kb ($p \leq 0.01$) and 4 – 5 kb ($p \leq 0.05$) than that of the males (Fig 3.7). At 8 months old, female *O. melastigma* still exhibited a lower proportion of short TRFs (< 2 kb) ($p \leq 0.01$), but a higher proportion of long TRFs (> 5 kb) ($p \leq 0.01$) than that of the males (Fig 3.7). At 12 months old, no significant gender difference was observed for any proportion of TRFs

(Fig 3.7).

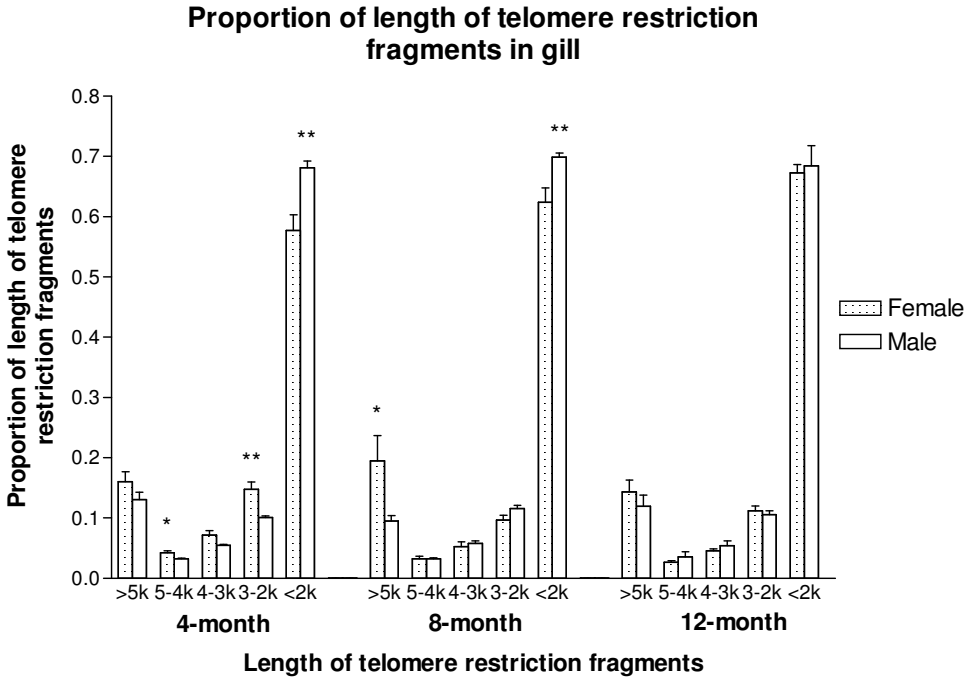


Fig 3.7 Change in the proportion of telomere restriction fragments (TRF from < 2 kb to > 5 kb in length) in the gills of male and female *Oryzias melastigma* at different ages (4 to 12 months old). Data are expressed as mean ± standard error of mean (n = 5). Significant differences between the sexes are indicated by asterisks (*, $p \leq 0.05$; **, $p \leq 0.01$).

3.7 Telomerase activity and TERT mRNA expression in liver

In the liver, telomerase activity (TA) was significantly higher in female *O. melastigma* than in that of the males at 4 months ($p \leq 0.001$) and 8 months old ($p \leq 0.001$), whereas no significant gender difference was detected in liver TA at 12 months old (Fig 3.8a). In females, liver TA peaked at 8 months old; in males it

levelled off after 8 months old (Kruskal-Wallis One-Way ANOVA, $p \leq 0.001$) (Fig 3.8a).

Consistent with the TA findings, liver TERT mRNA expression in *O. melastigma* was significantly higher in females than in males at 4 months ($p \leq 0.001$) and 8 months old ($p \leq 0.001$), whereas no significant gender difference was found in liver TERT mRNA at 12 months old (Fig 3.8b). In both females and males, liver TERT transcript expression peaked at 8 months (Kruskal-Wallis One-Way ANOVA, $p \leq 0.001$) (Fig 3.8b).

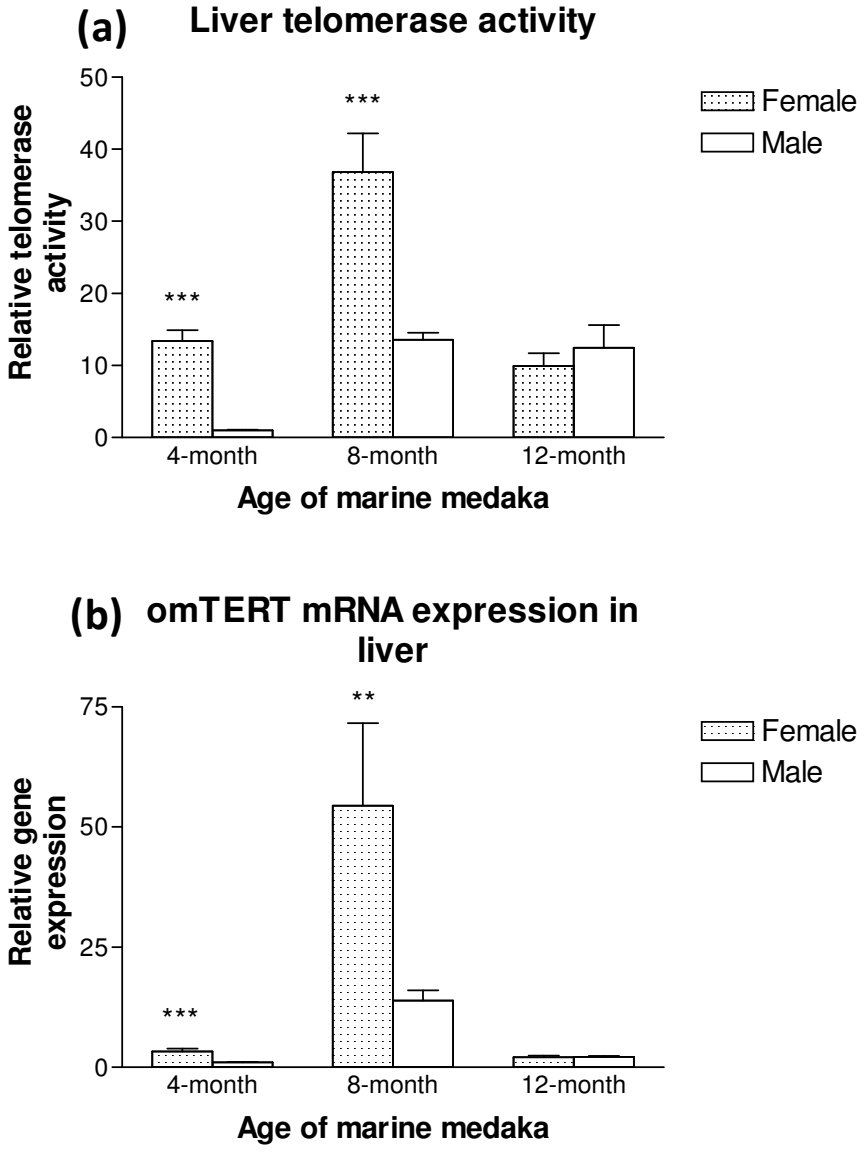


Fig 3.8. Age and gender difference in liver telomerase activity (a) and omTERT mRNA (b) in marine medaka *Oryzias melastigma* at 4 months, 8 months and 12 months old. Data are expressed as mean ± standard error of mean (n = 15). Significant differences between gender are indicated by asterisks (**, $p \leq 0.01$; ***, $p \leq 0.001$).

3.8 Telomerase activity and TERT mRNA expression in gill

In the gills, TA in *O. melastigma* was significantly higher in females than in males at 4 months ($p \leq 0.05$) and 8 months old ($p \leq 0.001$), whereas no significant gender difference was detected in 12-month old fish (Fig 3.9a). In female, gill TA was peak at 8-month old (Kruskal-Wallis One-Way ANOVA, $p \leq 0.001$). Gill TA dropped at 12-month in both the females (Kruskal-Wallis One-Way ANOVA, $p \leq 0.001$) and the males (Kruskal-Wallis One-Way ANOVA, $p = 0.005$) (Fig 3.9a).

However, no significant gender difference in gill TERT mRNA expression was observed in marine medaka at any age group studied (Fig 3.9b). In both females and males, gill TERT transcript expression peaked at 8 months (Kruskal-Wallis One-Way ANOVA, $p \leq 0.001$) (Fig 3.9b).

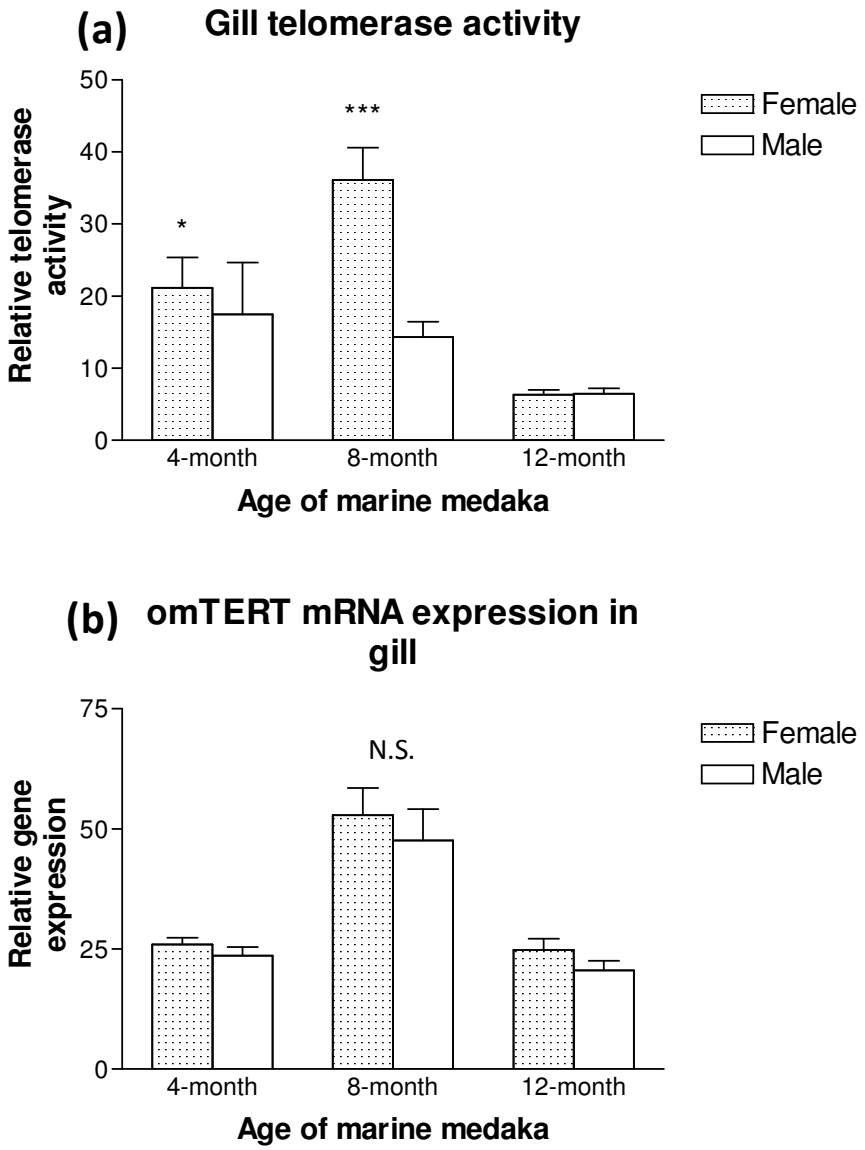


Fig 3.9 Age and gender difference in gill telomerase activity (a) and omTERT mRNA (b) in marine medaka *Oryzias melastigma* at 4 months, 8 months and 12 months old. Data are expressed as mean ± standard error of mean (n = 15). Significant differences between gender are indicated by asterisks (*, $p \leq 0.05$; ***, $p \leq 0.001$). N.S. = no significant difference.

3.9 ERα mRNA expression in liver and gill

In the liver, ERα mRNA expression was significantly higher in female than in male *O. melastigma* at all age groups studied ($p \leq 0.001$) (Fig 3.10). In the females, liver ERα mRNA expression peaked at 8 months and levelled off thereafter (Kruskal-Wallis One-Way ANOVA, $p \leq 0.001$) (Fig 3.10). In the males, there was no significant difference in ERα mRNA expression among age groups (Kruskal-Wallis One-Way ANOVA, $p > 0.05$) (Fig 3.10).

In the gills, ERα mRNA expression was close to undetectable in both males and females (Fig 3.10).

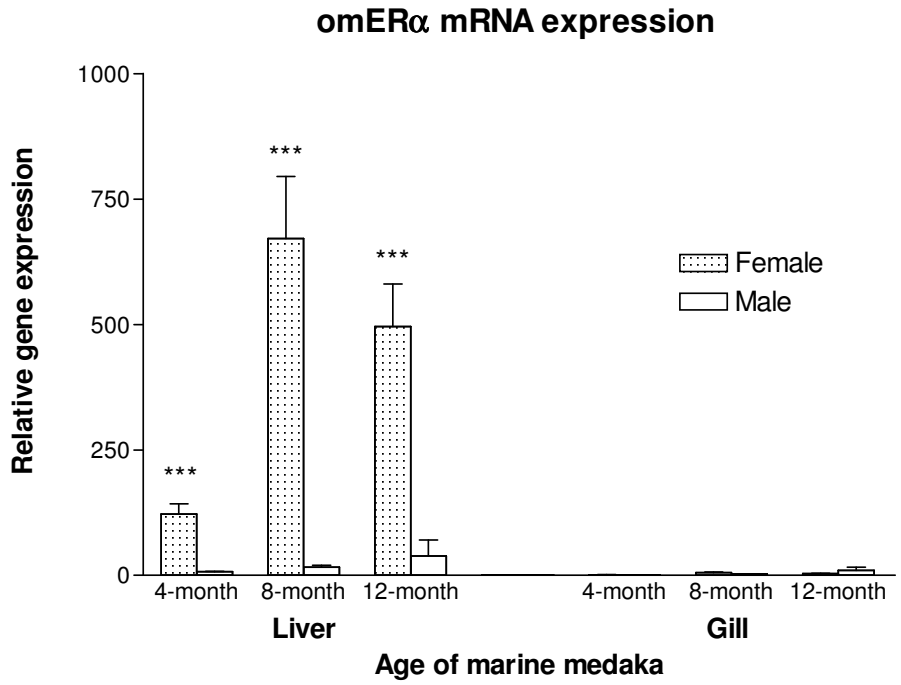


Fig 3.10 Age and gender difference in omERα mRNA in the liver and gills of marine medaka *Oryzias melastigma* at 4 months, 8 months and 12 months old. Data are expressed as mean ± standard error of mean (n = 15). Significant differences between gender are indicated by asterisks (***, $p \leq 0.001$).

3.10 DNA oxidation (8-OHdG level) in *O. melastigma*

In the liver, 8-OHdG level in *O. melastigma* was significantly lower in females than in males at 12 months old ($p \leq 0.05$), while no significant gender difference was detected in the liver of *O. melastigma* at 4 months and 8 months old (Fig 3.11a). In males, liver 8-OHdG level peaked at 12 months old (Kruskal-Wallis One-Way ANOVA, $p \leq 0.05$), whereas it gradually increased from 4 months to 12 months in the females (Kruskal-Wallis One-Way ANOVA, $p \leq 0.05$) (Fig 3.11a).

In the gills, 8-OHdG level was significant lower in females than in males at 8 months old ($p \leq 0.05$), while no significant gender difference was observed in the gills of marine medaka at 4 months and 12 months old (Fig 3.11b). In both females and males, gill 8-OHdG level remained stable from 4 months to 12 months (Kruskal-Wallis One-Way ANOVA, $p > 0.05$) (Fig 3.11b).

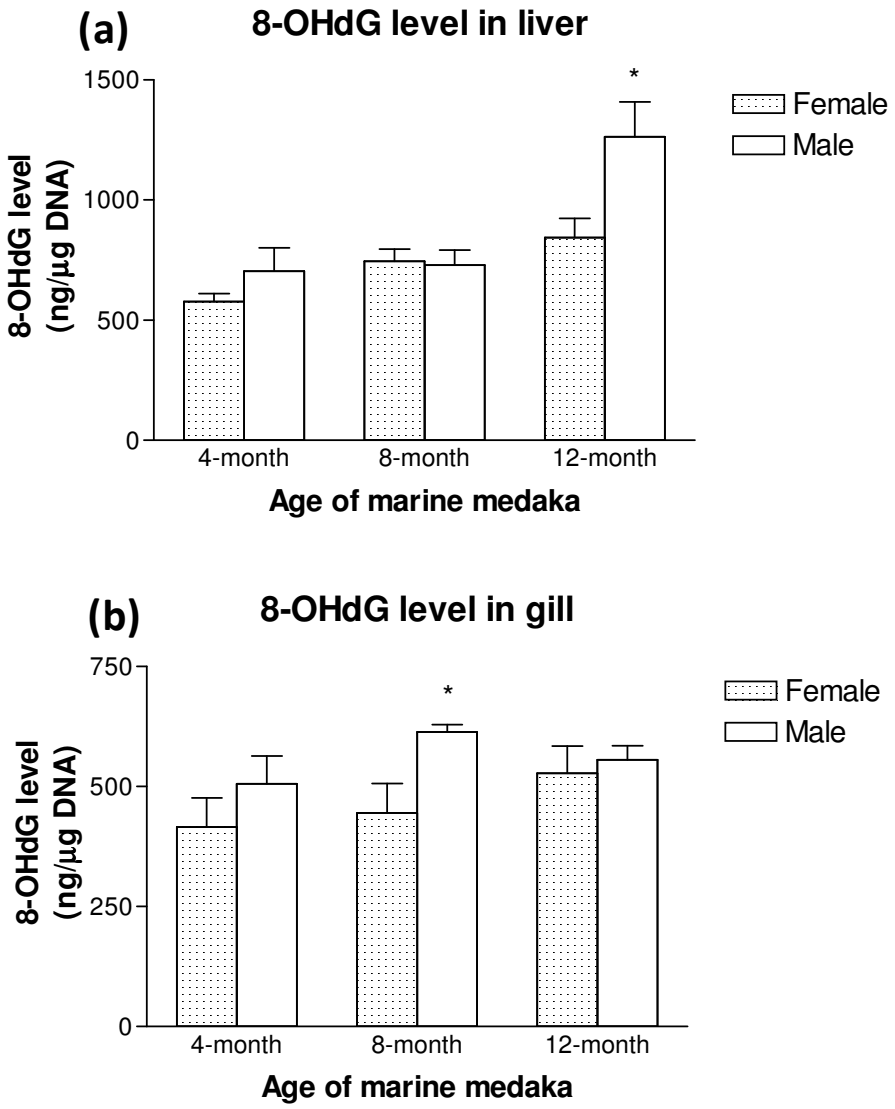


Fig 3.11 Age and gender difference in 8-OHdG level in the liver (a) and the gills (b) of marine medaka *Oryzias melastigma* at 4 months, 8 months and 12 months old. Data are expressed as mean ± standard error of mean (n = 5). Significant difference between gender is indicated by asterisk (*, $p \leq 0.05$).

3.11 Protein oxidation (protein carbonyl level) in *O. melastigma*

In the liver, the protein carbonyl level in *O. melastigma* was significantly lower in females than in males at 8 months ($p \leq 0.05$) and 12 months old ($p \leq 0.01$), while no significant gender difference was observed in *O. melastigma* at 4 months old (Fig 3.12a). In females, the liver protein carbonyl level levelled off at 8 months and 12 months old, whereas it gradually increased from 4 months to 12 months in males (Kruskal-Wallis One-Way ANOVA, $p \leq 0.01$) (Fig 3.12a).

In the gills, the protein carbonyl level in *O. melastigma* was significantly lower in females than in males at 8 months ($p \leq 0.01$), while no significant gender difference was observed in *O. melastigma* at 4 months and 12 months old (Fig 3.12b). In females, the gill protein carbonyl level remained stable from 4 months to 12 months, whereas it gradually dropped from 4 months to 12 months in males (Kruskal-Wallis One-Way ANOVA, $p \leq 0.05$) (Fig 3.12b).

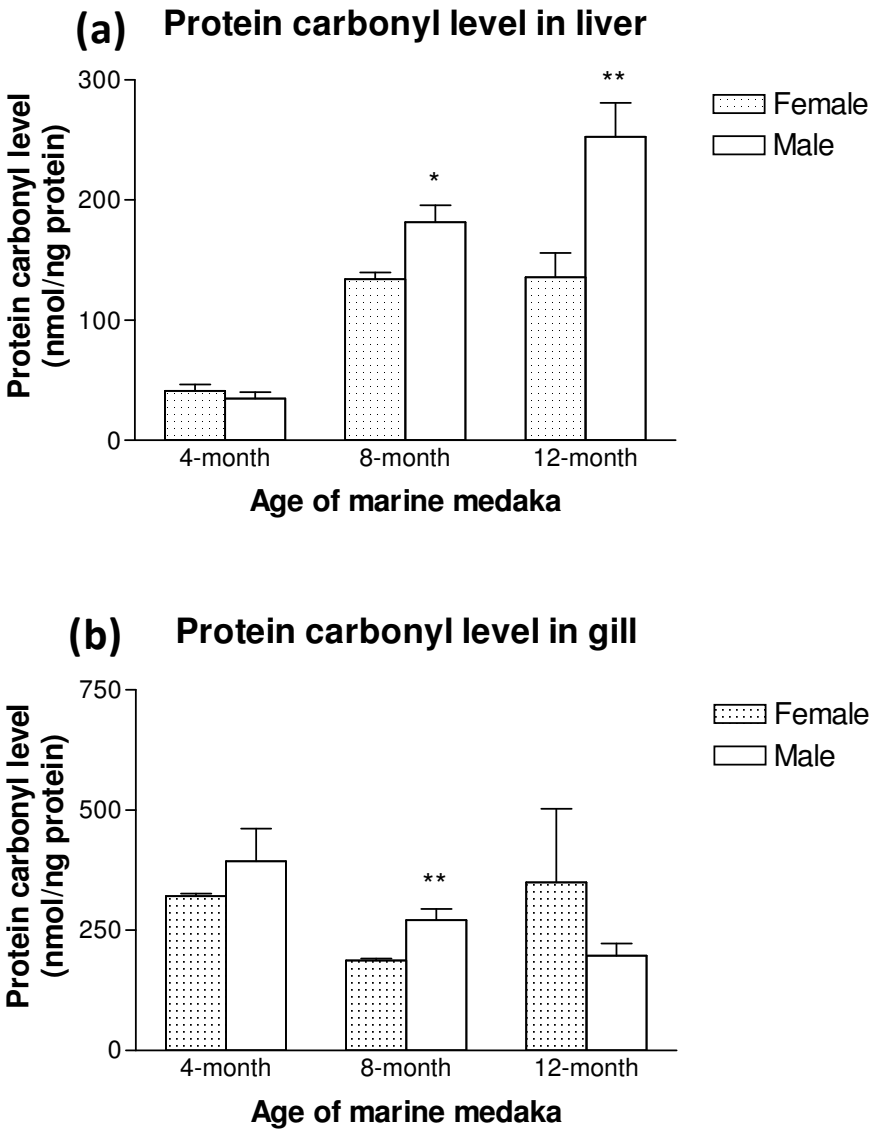


Fig 3.12 Age and gender difference in the protein carbonyl level in the liver (a) and gills (b) of marine medaka *Oryzias melastigma* at 4 months, 8 months and 12 months old. Data are expressed as mean ± standard error of mean (n = 5). Significant difference between gender is indicated by asterisk (*, $p \leq 0.05$; **, $p \leq 0.01$).

3.12 Correlations between plasma E2 level and telomerase activity in liver and gill

The results of Spearman rank order correlation analysis show that the level of plasma E2 in *O. melastigma* (including male and female fish at all age groups) was positively correlated with telomerase activity in the liver ($r = 0.224$; $p \leq 0.05$) as well as in the gills ($r = 0.383$; $p \leq 0.001$) (Table 3.2). The scatter plots are shown in Appendix 3.

Table 3.2 Spearman correlation coefficients (r) between plasma E2 level and telomerase activity in the liver and the gills of *O. melastigma* (n = 90).

	E2	
	Liver	**Gill**
Telomerase activity	0.224 *	0.383 ***

Significant correlations are indicated with asterisks (*, $p \leq 0.05$; ***, $p \leq 0.001$).

3.13 Correlations between plasma E2 level and mRNA expressions as well as telomerase activity in liver and gill

The results of Spearman rank order correlation analysis show that E2 was positively correlated with ERα mRNA expression in the liver ($r = 0.471$; $p \leq 0.001$) (Table 3.3). The scatter plot is shown in Appendix 3. As the expression of ERα transcripts was close to undetectable in the gills, no correlation analysis was

performed between gill ERα and E2/TA.

No significant correlation was found between E2 and TERT mRNA expression in either the liver or the gills of marine medaka ($p > 0.05$) (Table 3.3).

Telomerase activity was positively correlated with *TERT* expression in both the liver ($r = 0.649$; $p \leq 0.001$) and the gills ($r = 0.379$; $p \leq 0.001$), whereas significant correlation between telomerase activity and *ERα* expression was found only in the liver ($r = 0.489$; $p \leq 0.001$) (Table 3.3). The scatter plots are shown in Appendix 3.

Table 3.3 Spearman correlation coefficients (r) among plasma E2 level, telomerase activity, TERT mRNA expression and ERα mRNA expression in the liver and the gills of marine medaka (n = 90).

	E2		Telomerase activity	
	Liver	**Gill**	**Liver**	**Gill**
ERα	0.471 ***	-	0.489 ***	-
TERT	N.S.	N.S.	0.649 ***	0.379 ***

Significant correlations are indicated with asterisks (***, $p \leq 0.001$).
N.S.: not significant
"-" not available

3.14 Correlations among plasma E2, 8-OHdG and protein carbonyl levels in liver and gill

The results of Spearman rank order correlation analysis show that E2 level was negatively correlated with 8-OHdG level ($r = -0.436$; $p \leq 0.05$) in both the liver and the gills ($r = -0.542$; $p \leq 0.01$) (Table 3.4). A significant negative correlation between E2 and protein carbonyl level was detected only in the liver ($r = -0.636$; $p \leq 0.001$) (Table 3.4). The scatter plots were shown in Appendix 3.

Table 3.4 Spearman correlation coefficients (r) among plasma E2, 8-OHdG and protein carbonyl levels in the liver and the gills of marine medaka (n = 30).

	E2	
	Liver	**Gill**
8-OHdG	-0.436 *	-0.542 **
Protein carbonyl	-0.636 ***	N.S.

Significant correlations are indicated with asterisks (*, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$).
N.S.: not significant

CHAPTER 4 DISCUSSION

4.1 Longevity gender gap (LGG) in *Oryzias melastigma*

The present study is the first to show that LGG exists in *O. melastigma*, i.e. females live longer than males. This is consistent with the limited reports done on other fish (Krumholz 1948; Ketchen 1975; Vargas and de Sostoa 1996; Cabral and Marques 1999; Castilho et al. 2003). The phenomena of LGG (females living longer than males) appears to be universal in most vertebrates and not limited to humans (Appendixes 1 and 2; Vina et al. 2003, 2005; Stindl 2004; Aviv et al. 2005; Vina and Borras 2010) and other mammals (Asdell et al. 1967; Austad 1997; Allman et al. 1998; Coulson et al. 2001; Hill et al. 2001; Richard et al. 2002; Vina et al. 2003, 2005; Vina and Borras 2010) as well as non-mammals (Krumholz 1948; Das 1959; Liles and Delong 1960; Tinkle 1972, 1973; Scott 1974; Ketchen 1975; Ballinger and Congdon 1981; Ryan et al. 1983; Strijbosch and Creemers 1988; Holldobler and Wilson 1990; Lints et al. 1993; de Carvalho and Vasconcellos 1995; le Bourg and Minois 1996; Vargas and de Sostoa 1996; Nuzhdin et al. 1997; Wedekind and Jakobsen 1998; Cabral and Marques 1999; Castilho et al. 2003 Marzona et al. 2004; Davies et al. 2005; Lauck 2005; Keller and Jemielity 2006; Jemielity et al. 2007; Maklakov et al. 2007; Grabherr et al. 2009; reviewed in Austad 2006).

The female *O. melastigma* has begun to outnumber the males by the time they reach 10-months old (Fig 3.1). The maximum lifespan of *O. melastigma* reared under laboratory conditions is approximately 18 months (Au, unpublished data) while that of humans is known to be 123 years (Hayflick 2007). Assuming that the lifespan of *O. melastigma* can be scaled up to match human age, by direct conversion, an *O. melastigma* at 10 months old is equivalent to a human at 68 years old. This is similar to the situation in humans when at the age of 65 or above women already outnumber men in nearly all countries (Appendix 1), and the sex ratio (males/females) of humans over 65 years old in the world was 0.79 in 2010 (The World Factbook). In the same way, an *O. melastigma* at 12 months old is equivalent to a human at 82 years old and the sex ratio of people in the world over 80 years old was 0.64 (World Population Ageing 2009, Population Division, Department of Economic and Social Affairs, UN), which is very similar to the sex ratio of 12-month old *O. melastigma*, 0.63 (Table 3.1). It is acknowledged that the reproducibility of this part of our work needs further confirmation because of natural variability and/or batch-to-batch variations of stock culture. Nevertheless, the present findings showing males dying faster in *O. melastigma* than in humans may be partly explained by the fact that *O. melastigma* in general have more short telomeres than humans (Figs 3.4 – 3.7).

Table 4.1 summarizes the plasma E2 levels reported in fish. It is noted that the

E2 levels vary dramatically not only among species, but also between gender and age of the same species. Difference in the methods used for E2 measurement (ELISA vs RIA) may also contribute to the observed variations, rendering it difficult to make direct comparison of plasma E2 level among fish species. For instance, the plasma E2 levels in Japanese medaka *Oryzias latipes*, a close relative of *O. melastigma*, could be similar to or 750x higher than that of *O. melastigma*. In female *O. melastigma,* plasma E2 level was highest at 8 months old (18.08 ng/ml) and lowest at 12 months old (1.44 ± 0.28 ng/ml) which was similar to the E2 levels in male *O. melastigma* (0.54 – 2.04 ng/ml). The dramatic drop of E2 in 12-month *O. melastigma* may be due to a decrease in the function of ovaries (Reznick et al. 2006). This was evident by a decline in number of egg production in 12-months old fish (unpublished data of Rudolf Wu's group) as well as a significant decrease in gonadosomatic index (GSI) and a x1.5 times reduction of vitellogenic oocytes in female *O. melastigma* from 4 months to 12 months (Figs 4.1 – 4.2; unpublished data of Doris Au's group).

The maximum lifespan of humans and selected fish (listed in Table 4.1) also varies dramatically (Table 4.3). The maximum lifespan of *O. melastigma* and *O. latipes* was 18 months (under lab. condition) and 5 years (captivity) respectively (Table 4.3). These two medaka species exhibited relatively high plasma E2 levels (*O. melastigma* 0.54 – 18.08 ng/ml; *O. latipes* 4 – 401 ng/ml) (Table 4.1) compared to

humans (0.01 – 0.14 ng/ml) (Table 4.2) and rainbow trout *Oncorhynchus mykiss* (0.51 ± 0.06 ng/ml) (Tables 4.1). Given that the maximum lifespan of humans and rainbow trout was 123 years and 11 years respectively (Table 4.3), the findings suggest that plasma E2 level cannot be extrapolated to explain the maximum lifespan across species. It may at the most be related to longevity within a species (for example, the gender difference in plasma E2 level in *O. melastigma* is related to the gender difference in longevity of *O. melastigma*).

Table 4.1 Plasma E2 levels of fish reported in this study and other literatures.

Species	Age or size	Blood drawn from	Volume of blood collected (μl) per fish	No. of fish pooled	Method of measurement	E2 level in females (ng/ml)	E2 level in males (ng/ml)	Reference
Marine medaka (*Oryzias melastigma*)	4 months old	Aorta at spinal cord	2	3	ELISA	14.47 ± 1.07	2.04 ± 0.09	The present study[a]
	8 months old					18.08 ± 3.22	0.54 ± 0.05	
	12 months old					1.44 ± 0.28	0.74 ± 0.10	
Japanese medaka (*Oryzias latipes*)	Body length: 3 – 4 cm Body weight: 0.4 – 0.6 g	Caudal blood vessel	10 or less	10 – 20	Radioimmunoassay (RIA)	10 at 20h before spawning, 4 at 16h, 16 at 8h	N.A.	Soyano et al. 1993[a]
	Approximately 3 months old	Isthmus above heart		2	ELISA	401 ± 92, 363 ± 111, 133 ± 20, 263 ± 103*	225 ± 59, 239 ± 88, 56 ± 33, 247 ± 79*	Foran et al. 2002[a]
	Adults	Aorta at isthmus			ELISA	29.2 ± 13.9*	39.1 ± 15.3*	Tilton et al. 2003[b]
	Adults	Isthmus above heart		2	ELISA	230.7 ± 100.9*	229.4 ± 97.0*	Foran et al. 2004[a]
	4 months old	Aorta at isthmus			ELISA	~ 50*	~ 75*	Tilton et al. 2005[b]

Note: * indicates the data extracted from the control experiment(s) of the study, [a] indicates the data expressed as mean ± SEM, [b] indicates the data expressed as mean ± SD, N.A.: data not available

Table 4.1 (continued)

Species	Age or size	Blood drawn from	Volume of blood collected (µl) per fish	No. of fish pooled	Method of measurement	E2 level in females (ng/ml)	E2 level in males (ng/ml)	Reference
Zebrafish (*Danio rerio*)	18 weeks old	Caudal vein		8	ELISA	~ 0.75*	~ 0.25*	Liu et al. 2009[a]
Rainbow trout (*Oncorhynchus mykiss*)	1 year old	Caudal vasculature			RIA	0.51 ± 0.06*	N.A.	Nakamura et al. 2009[a]
Amago salmon (*Oncorhynchus rhodurus*)					RIA	3 – 110	N.A.	Kagawa et al. 1983
Blue tilapia (*Tilapia aurea*)					RIA	3.1 ± 0.7	N.A.	Yaron et al. 1977
Japanese whiting (*Sillago japonica*)	38 – 145 g	Caudal vessels			RIA	0.5 – 1.35	N.A.	Matsuyama et al. 1990
Paiche (*Arapaima gigas*)		Caudal puncture			ELISA	0.014 – 0.637	0.002 – 0.435	Chu-Koo et al. 2009
Protandrous seabass (*Lates calcarifer*)					RIA	0.598 ± 0.369	0.068	Guiguen et al. 1993

Note: * indicates the data extracted from the control experiment(s) of the study, [a] indicates the data expressed as mean ± SEM, [b] indicates the data expressed as mean ± SD, N.A.: data not available

Table 4.1 (continued)

Species	Age or size	Blood drawn from	Volume of blood collected (µl) per fish	No. of fish pooled	Method of measurement	E2 level of females (ng/ml)	E2 level of males (ng/ml)	Reference
White-spotted char (*Salvelinus leucomaenis*)	2 years old	Caudal vasculature			RIA	0.63 – 13.21	N.A.	Kagawa et al. 1981
Red sea bream (*Pagrus major*)					RIA	0.2 – 1.2	N.A.	Matsuyama et al. 1988

Note: N.A.: data not available

Table 4.2 Plasma E2 levels of humans.

E2 level of females (ng/ml)		E2 level of males (ng/ml)		Method of measurement	Reference
Days 1 – 10 of menstrual cycle	0.0656				
Days 11 – 20 of menstrual cycle	0.1245		0.024	RIA	Korenman et al. 1969
Days 21 – 31 of menstrual cycle	0.1372				
Post-menopausal women	0.015				
0.095 ± 0.0069		0.017 ± 0.00071		RIA	Jenner et al. 1972
Pre-menopausal women	0.015 – 0.35				
Post-menopausal women	> 0.01		0.01 – 0.04	LC-MS/MS	Nelson et al. 2004
Pre-pubescent girls	0.000490 - 0.0178	Pre-pubescent boys	0.000490 – 0.00460	GC-MS/MS	Courant et al. 2010

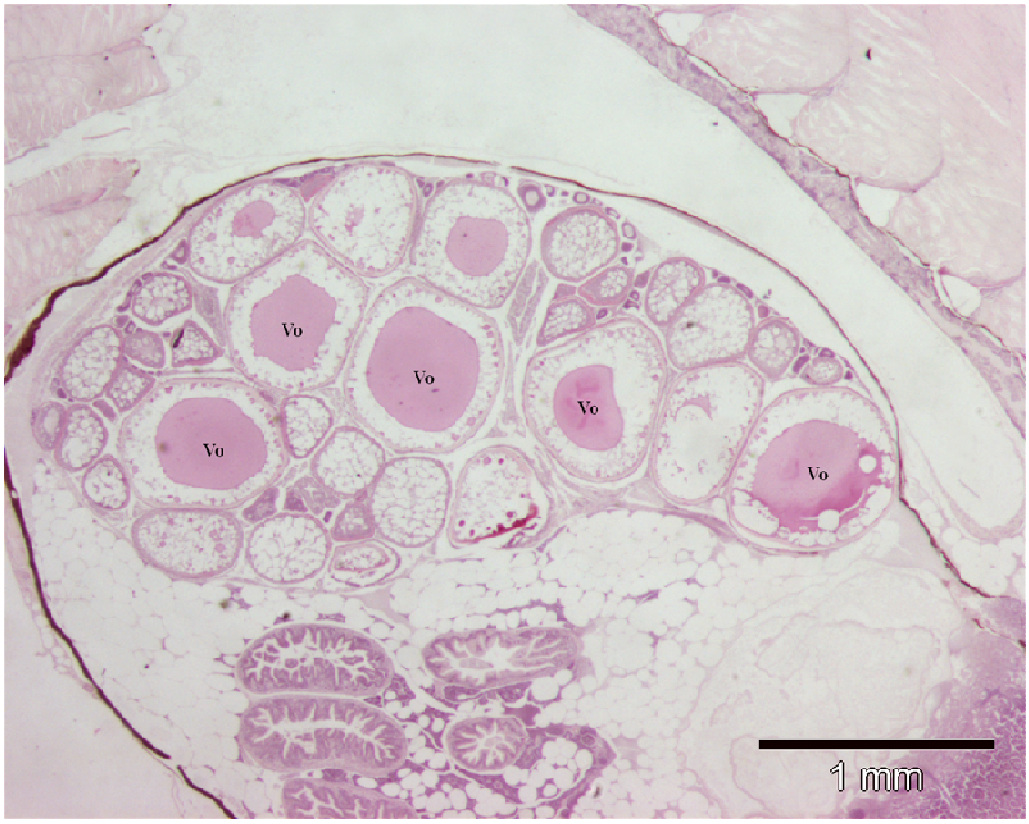


Fig 4.1 Representative histological section of the ovary of 4-months old *O. melastigma*, hematoxylin and eosin staining. Vo = vitellogenic oocyte. Scale bar = 1mm.

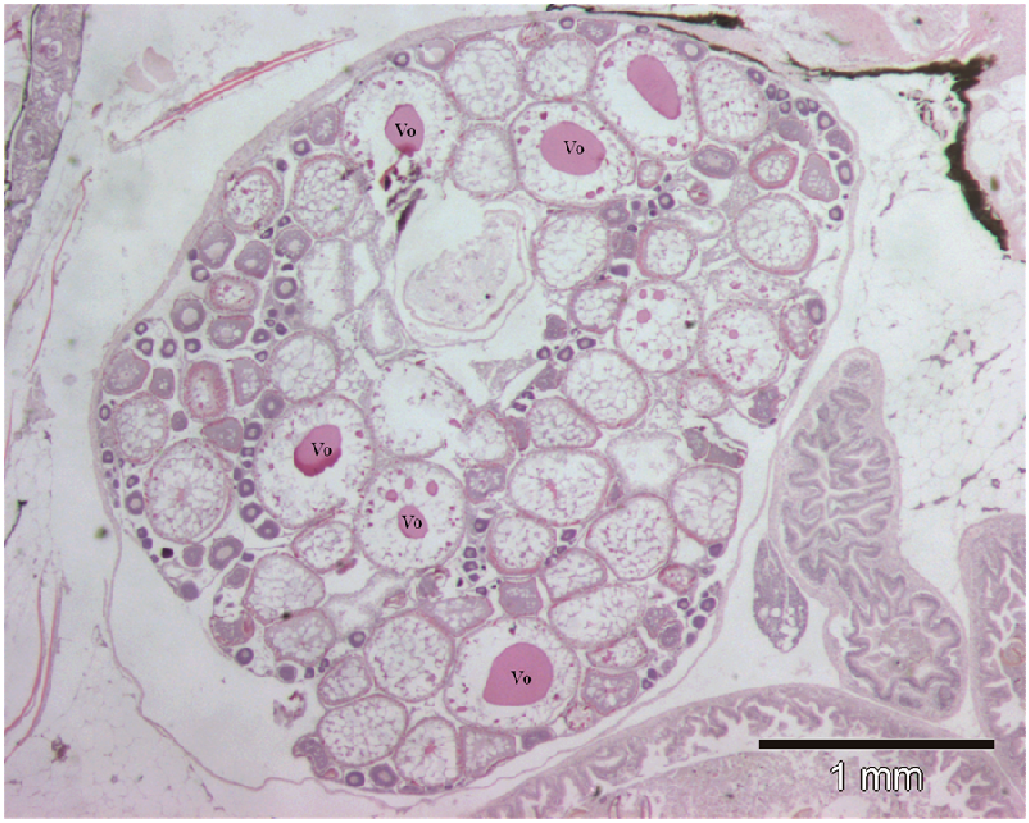


Fig 4.2 Representative histological section of the ovary of 12-months old *O. melastigma*, hematoxylin and eosin staining. Vo = vitellogenic oocyte. Scale bar = 1mm.

Table 4.3 Maximum lifespan of humans and selected fish listed in Table 4.1.

Species	Maximum lifespan	Reference
Marine medaka (*Oryzias melastigma)*	18 months	Au, unpublished data
Japanese medaka (*Oryzias latipes*)	5 years (captivity)	Egami 1971
Zebrafish (*Danio rerio*)	5.5 years (captivity)	Gerhard et al. 2002
Rainbow trout (*Oncorhynchus mykiss*)	11 years (wild)	FishBase (www.fishbase.org)
Blue tilapia (*Tilapia aurea*)	8 – 11 years	Riedel et al. 2002
Protandrous seabass (*Lates calcarifer*)	20 years	Balston 2009
Humans	123 years	Hayflick 2007

In vitro studies using human cell cultures (Table 4.4) showed that human cells seldom contain mean telomere restriction fragment (TRF) length < 4 kb. The presence of TRF < 4kb length was correlated with the induction of replicative senescence or apoptosis *in vitro* (Counter et al. 1992, 1994, 1998; Shay et al. 1993; Klingelhutz et al. 1994; von Zglinicki et al. 1995; Harley et al. 2011; reviewed in Calado and Young, 2009). However, unlike mammalian cells, the present study and Au et al. (2009) clearly showed that fry, young and middle-aged adult *O. melastigma* possess large portion of TRFs with length < 2 kb. In the liver and gills of 4-month to 12-month old *O. melastigma*, over 50 % of TRFs was found to be < 2 kb in length (Figs 3.4 – 3.7). However, significant replicative senescence did not occur in *O. melastigma* even though their cells contain a large portion of shorter telomeres (< 2 kb). This is evident

by the fact that both the *in vivo* growth parameters, body length and body weight, increased from young to middle-aged *O. melastigma* (with TRFs < 2kb) (Fig 3.2), indicating tissue turnover and fish growth had not been hindered. The above findings suggest that the critical TL triggering cellular senescence cannot be generalized, which is likely to vary among cells/tissues/animal types *in vitro* or *in vivo*. This has complicated studies on the direct involvement of TL shortening on replicative senescence.

Table 4.4 Human cell culture(s) and TL measurement methods used for the determination of mean TRF length < 4 kb that would induce replicative senescence or apoptosis.

Cell culture(s)	TL measurement method(s)	Reference
Human embryonic kidney cells	Southern blotting	Counter et al. 1992
Human mammary epithelial cells	Southern blotting	Shay et al. 1993
Human B lymphocytes	Southern blotting	Counter et al. 1994
Human anogenital epithelial cells	Southern blotting	Klingelhutz et al. 1994
Human WI-38 fibroblasts	Southern blotting	Von Zglinicki et al. 1995
Human embryonic kidney cells	Southern blotting	Counter et al. 1998
Peripheral lymphocytes and granulocytes	Flow fluorescence *in situ* hybridization (FlowFISH), quantitative polymerase chain reaction (qPCR), High-throughput quantitative fluorescence *in situ* hybridization (HT qFISH)	Harley et al. 2011

The plasma E2 level in *O. melastigma* was at least an order of magnitude higher than that in humans (Fig 3.3; Tables 4.1 – 4.2). The lowest plasma E2 level detected in 8-month old male *O. melastigma* (0.54 ± 0.05 ng/ml) was 1.5x higher than that in pre-menopausal women (0.35 ng/ml) (Table 4.2). The highest plasma E2 level in 8-month old female *O. melastigma* (18.08 ± 3.22 ng/ml) was 50x higher than that in pre-menopausal women (Tables 4.1 – 4.2). It is speculated that a relatively high plasma E2 level in young and middle-aged adult *O. melastigma* may help maintain the short telomeres from further erosion (Aviv 2002; Borras et al. 2003, 2007; Vina et al. 2003, 2005; Aviv et al. 2005; Lee et al. 2005; Mayer 2006; Bayne et al. 2007; Guan et al. 2007; Bayne et al. 2008; Vina and Borras 2010). The mechanisms by which estradiol protects telomeres in the liver and gills will be discussed in detail in Sections 4.5 and 4.6.

4.2 Sexual size dimorphism

Sexual size dimorphism is proposed as one of the possible explanations for LGG (Stindl 2004). However, even though there is no significant gender difference in the body length of *O. melastigma* at 4 months, 8 months and 12 months old (Fig 3.2a), the body weight of 4-month and 12-month old female medaka was heavier than their male counterparts (Fig 3.2b). It is clear that female *O. melastigma* are larger in size

than the male fish. If LGG in *O. melastigma* is due to sexual dimorphism of size, it should be the males that live longer. Therefore, sexual size dimorphism does not account for LGG in *O. melastigma*. In addition, female spiny dogfish, mosquitofish, frogs, newts, ants, tea red spiders and the giant kidney-worms in the maned wolf have a larger body size and greater longevity than males (Krumholz 1948; Das 1959; Ketchen 1975; Ryan et al. 1983; de Carvalho and Vasconcellos 1995; Vargas and de Sostoa 1996; Cabral and Marques 1999; Marzona et al. 2004; Lauck 2005; Keller and Jemielity 2006). Given the wide spectrum of controversial findings, sexual dimorphism of size is not a plausible explanation for LGG in animals.

4.3 Telomere length (TL) declined over age

With advancing age, the proportion of short TRFs length (< 2 kb) increased in the liver of female and male medaka and in the gills of female medaka (Figs 3.4, 3.6). Generally the above findings are consistent with previous studies showing that TL declines with age *in vitro* (Harley et al. 1990; de Lange et al. 1990; Hastie et al. 1990; Lindsey et al. 1991) and *in vivo* (Harley et al. 1990; Vaziri et al. 1993; Coviello-McLaughlin and Prowse 1997; Okuda et al. 2000; Haussmann and Vleck 2002; Tsuji et al. 2002; Baerlocher et al. 2003, 2007; Scott et al. 2006; Guan et al. 2007; Flores et al. 2008; Hatakeyama et al. 2008; Anchelin et al. 2011). However, no

progressive shortening of TL was observed in the gills of male *O. melastigma* (Fig 3.6). This could be due to the fact that the gills of young males already contain a large proportion of telomeres with length < 2 kb (68 %) at a very young age, 4 months old.

In humans, telomerase activity has been detected only in regenerative and reproductive tissues, but was undetectable in most somatic cells/tissues (Morin 1989; Kim et al. 1994; Wright et al. 1996; Shay and Bacchetti 1997; Cong et al. 2002; Flores et al. 2006). In fish, however, TA together with TERT mRNA and protein were ubiquitously expressed in somatic, reproductive and regenerative tissues (Table 4.5) (Klapper et al. 1998; Yoda et al. 2002; Kishi et al. 2003; McChesney et al. 2005; Yap et al. 2005; Yip et al. 2005; Yu et al. 2006; Lau et al. 2007, 2008; Pfennig et al. 2008; reviewed in Mok 2008). The ubiquitous expression of TA may be essential in maintaining the short telomeres in cells of *O. melastigma*. However, it remains unclear why a higher TA in the gills of young and middle-aged female *O. melastigma* (Fig 3.9a) failed to maintain TL, compared to the male counterparts (Fig 3.6). This may be related to higher oxidative stress in the gills, which have lower antioxidant potential than the liver (Sayeed et al. 2003; Oberdorster 2004; Monteiro et al. 2006).

Table 4.5 Tissue distribution of TERT mRNA, protein expression and telomerase activity (TA) in various fish species (modified from Mok 2008).

Organism	TERT mRNA	TERT protein	TA	Reference
Marine medaka (*Oryzias melastigma*)	Liver and gills		Liver and gills	The present study
	Liver, gills, brain, heart, spleen, kidney, intestine, eye, muscle, skin, ovary and testis			Yu et al. 2006
	Liver, gills, brain, kidney, intestine, muscle, ovary and testis	Liver, gills, brain, kidney, intestine, muscle, ovary and testis		Kong et al. 2008
			Liver, gills, brain, heart, spleen, kidney, intestine, eye, muscle, skin and gonad	Au et al. 2009
Japanese medaka (*Oryzias latipes*)	7-month old adult: Liver, gills, brain, heart, spleen, kidney, muscle, skin, ovary and testis		Adult female and male: Brain, heart, ovary and testis	Pfennig et al. 2008
American eel (*Anguilla rostrata*)			Stomach, intestine, gall bladder and spleen	McChesney et al. 2005

Table 4.5 (continued)

Organism	TERT mRNA	TERT protein	TA	Reference
Goldfish (*Carassius auratus*)	Adult: Retina	Adult: Ganglion cell layer, inner and outer nuclear layer	Adult: Retina	Lau et al. 2007
Pufferfish (*Fugu rubripes*)	Gills, brain, heart, stomach, spleen, skin, ovary, testis, eye and fugu eye derived cells			Yap et al. 2005
Rainbow trout (*Oncorhynchus mykiss*)			Liver, brain, heart, kidney, muscle and skin	Klapper et al. 1998
			Eyed embryos	Yoda et al. 2002
Zebrafish (*Danio rerio*)			5-month, 15-month and 24-month old adult: Muscle	Kishi et al. 2003
			Eggs, brain, eye and muscle	McChesney et al. 2005
	Brain and retina	Brain and retina	Brain and retina	Yip et al. 2005
	Liver, gills, brain, retina, heart, kidney, muscle, skin and ovary	Ganglion cell layer, inner and outer nuclear layer	Gills, brain, retina, heart, kidney, muscle, skin and ovary	Lau et al. 2008

An absence of progressive shortening of TL detected in the gills of aging male medaka (Fig 3.6) could be due to the fact that Southern blotting was not sensitive enough to detect subtle differences in TRF lengths (Canela et al. 2007; Anchelin et al. 2011). Moreover, TRF lengths may vary due to sub-telomeric restriction site polymorphisms and sub-telomeric length polymorphisms (Cawthon 2002; Lin and Yan 2005) (Fig 4.3). For short TRF lengths in particular, a highly variable sub-telomeric region may easily affect the accuracy of TL measurement. More sensitive techniques such as Quantitative Fluorescence *In Situ* Hybridization (Q-FISH) are promising for detecting precise telomere length (Anchelin et al. 2011). Q-FISH is a technique which allows the hybridization of telomeric DNA with telomere repeat oligonucleotide probes labeled with fluorescent dye such as cyanine 3 (Cy3) or fluorescein isothiocyante (FITC) on cells or tissue sections. The number of telomere repeats on each telomere can be quantified based on the intensity of fluorescent signal on targets, detected using a highly sensitive digital imaging system attached to a fluorescence or confocal laser microscope (Lansdorp et al. 1996; Hultdin et al. 1998; Slijepcevic 1998). Importantly, the influence of sub-telomeric regions can be excluded by Q-FISH.

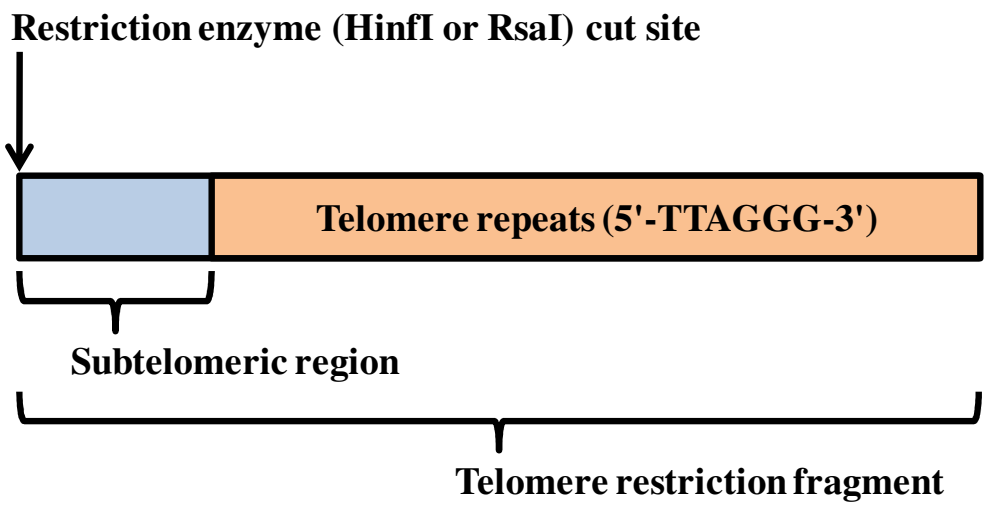


Fig 4.3 TRF lengths may vary due to sub-telomeric length polymorphism.

4.4 Gender difference in TL and E2

Gender differences in the proportion of TRFs with length < 2 kb (F < M) were observed in the liver and gills of young and middle-aged *O. melastigma* (Figs 3.5 and 3.7). This is in line with mammalian studies showing longer TL in females than males (Coviello-McLaughlin and Prowse 1997; Jeanclos et al. 2000; Benetos et al. 2001; Cawthon et al. 2003; Cherif et al. 2003; Mayer 2006; Guan et al. 2007). In leukocytes of human newborns, there was no statistically significant gender difference in TL (Okuda et al. 2002). Cross-sectional population analyses indicate that the age-adjusted TL of pre-menopausal women is longer than that of similarly aged men (Jeanclos et al. 2000; Benetos et al. 2001; Cawthon et al. 2003; Mayer 2006; Guan et al. 2007). The findings suggest a gender difference in telomere erosion in humans (males > females),

and possibly in *O. melastigma*.

Gender difference in plasma E2 levels were detected in young and middle-aged *O. melastigma*, but no significant gender difference in E2 level was found in old *O. melastigma* (Fig 3.3), which was concomitant with the findings of TL in *O. melastigma.* E2 is likely the key factor leading to the gender difference in TL in *O. melastigma*, as suggested by other literature based on human studies (Aviv 2002; Aviv et al. 2005; Bayne et al. 2007).

Since it is well documented that E2 suppresses oxidative stress (Vina and Borras 2010) and oxidative damage causes telomere shortening (Petersen et al. 1998; Henle et al. 1999; Oikawa et al. 2001; von Zglinicki 2002; Kurz et al. 2004), it is likely that, in addition to E2 induction of TA, the suppression of oxidative damage by E2 is also responsible for repressing telomere shortening in *O. melastigma*. Here, it is proposed that E2 may prevent telomere erosion in tissues of *O. melastigma* via two major mechanisms: (1) the elevation in TA, and (2) the suppression of oxidative DNA damage on telomeres (Fig 4.4).

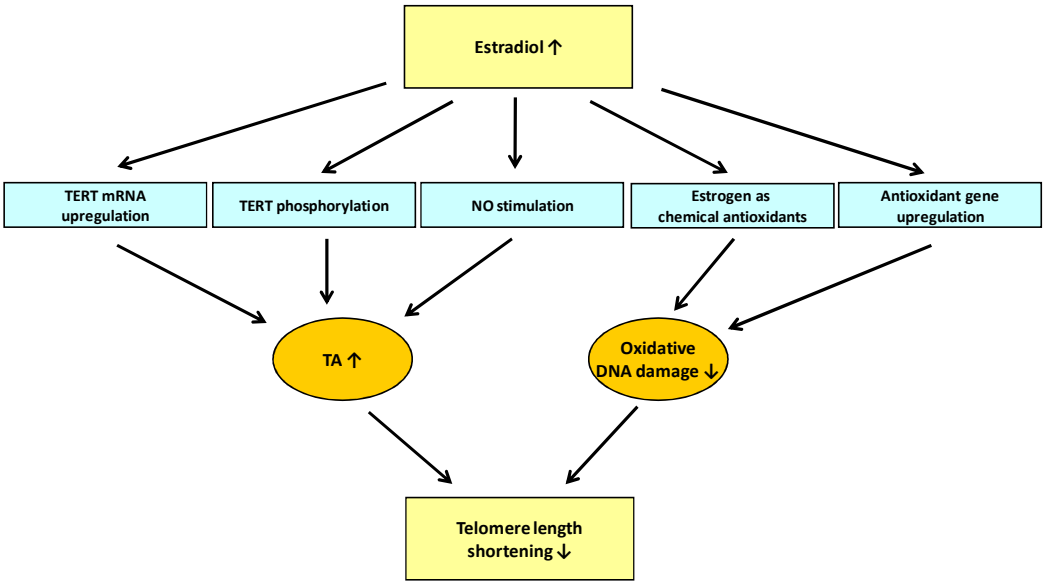


Fig 4.4 A schematic diagram showing the possible pathways of E2 mediated suppression of telomere length shortening.

Since the E2 up-regulation of TA via NO stimulation was exclusively demonstrated on mammalian endothelial cells (for review, see Zeng and Xu 2008) and estrogen as a chemical antioxidant was unfeasible at the very low physiological level (Vina et al. 2003, 2005; Vina and Borras 2010), these two E2 mediated pathways were excluded. The modified schematic diagram is shown in Fig 4.5.

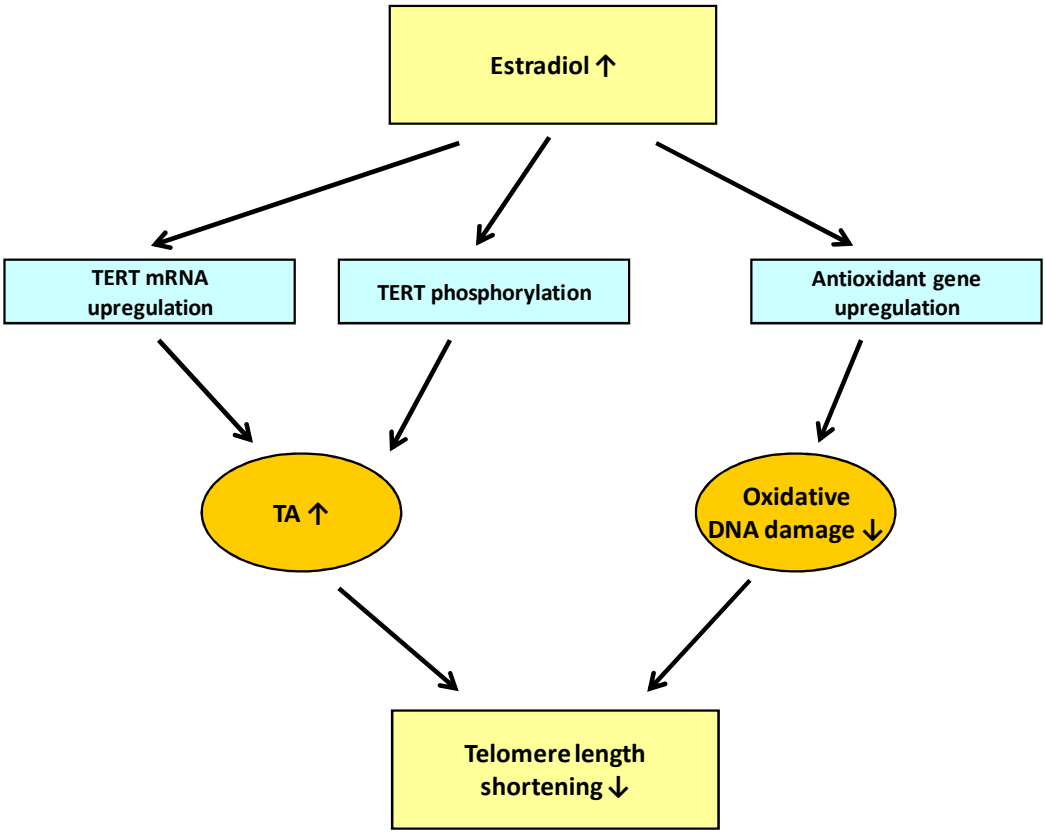


Fig 4.5 A modified schematic diagram showing the possible pathways of E2 mediated suppression of telomere shortening in *Oryzias melastigma*.

4.5 Relating TA, TERT and ERα mRNA expressions, and oxidative damage (8-OHdG and protein carbonyl levels) to E2 and TL in *O. melastigma* liver and gills

4.5.1 Possible mechanisms in the liver

Gender difference is clearly evident in plasma E2 level and liver TL, TA, TERT and ERα mRNA expressions, 8-OHdG and protein carbonyl levels in *O. melastigma* at 4 months to 12 months old (Table 4.6).

Table 4.6 Gender difference in plasma E2 level, telomere length (TL), telomerase activity (TA), TERT and ERα mRNA expressions, and 8-OHdG and protein carbonyl levels in the liver of *Oryzias melastigma* at 4 months to 12 months old.

Liver	Age		
	4 months (young)	8 months (middle-aged)	12 months (old)
Plasma E2 level	F > M ***	F > M ***	n.s.
TL (< 2 kb)	F < M *	F < M **	n.s.
TA	F > M ***	F > M ***	n.s.
TERT mRNA expression	F > M ***	F > M **	n.s.
ERα mRNA expression	F > M ***	F > M ***	F > M ***
8-OHdG	n.s.	n.s.	F < M *
Protein carbonyl	n.s.	F < M *	F < M **

*, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$; n.s. = no significant difference

Abundant *in vitro* and *in vivo* studies in mammals show that TA can be induced in ER-positive cells and tissues by E2 through the up-regulation of TERT gene transcription (Kyo et al. 1999; Misiti et al. 2000; Nanni et al. 2002; Kawagoe et al. 2003; Kimura et al. 2004; Imanishi et al. 2005; Boggess et al. 2006; Sarkar et al. 2006; Cha et al. 2008). In *O. melastigma,* plasma E2 was positively correlated with TA in the liver [Spearman correlation coefficients (r) = 0.224; $p \leq 0.05$] (Table 3.2). In addition, liver ERα mRNA expression was significantly higher in females than in

males at all age groups studied ($p \leq 0.001$) (Fig 3.10). This is in line with previous findings in other fish, e.g. channel catfish, European sea bass, rainbow trout, Japanese eel, mummichog and tilapia (Pakdel et al. 1989, 2000; Todo et al. 1996; Teves et al. 2003; Urushitani et al. 2003; Halm et al. 2004; Davis et al. 2008). In addition, liver ERα mRNA expression was positively correlated with plasma E2 level [Spearman correlation coefficients (r) = 0.471; $p \leq 0.001$] and liver TA [Spearman correlation coefficients (r) = 0.489; $p \leq 0.001$] (Table 3.3), suggesting ERα was involved in the up-regulation of TA by E2 in the liver.

Collectively, liver TA is highly likely to be activated through TERT gene transactivation by E2 interacting with ERα in *O. melastigma*, although other mechanisms of TA induction such as TERT protein phosphorylation cannot be excluded. The possible mechanisms in the liver are summarized in Fig 4.6 (blue box).

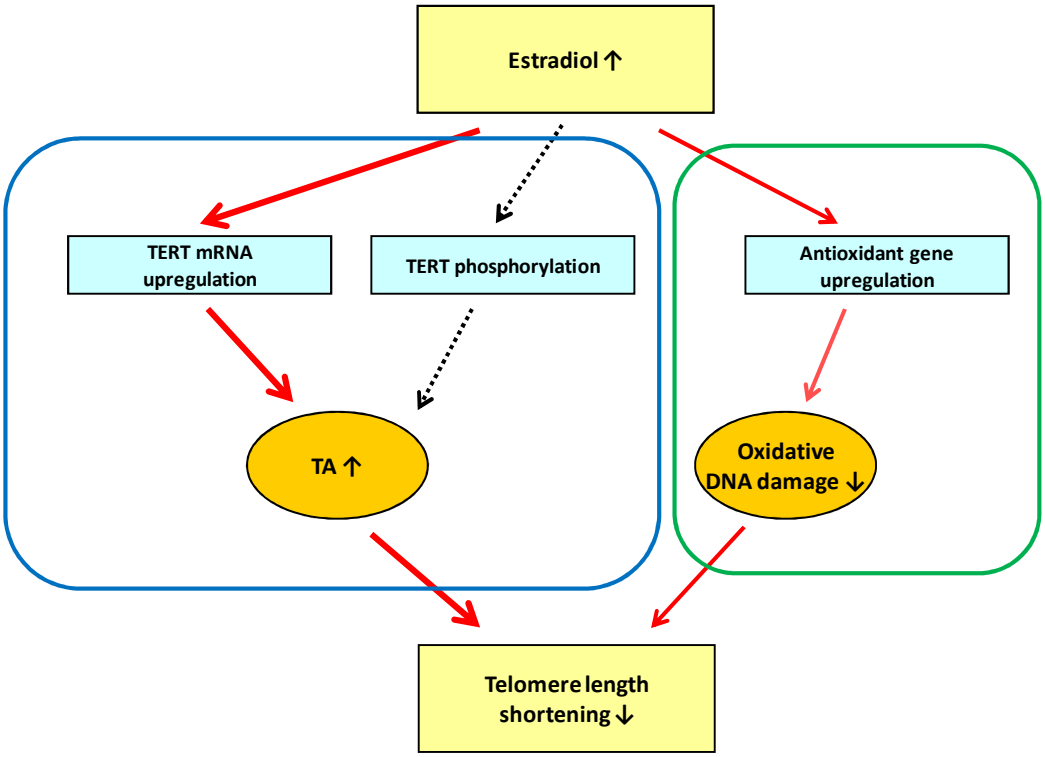


Fig 4.6 Possible mechanisms of E2 up-regulation of TA and oxidative DNA damage in the liver of *Orzyias melastigma*.

In the liver, increased DNA oxidation (as measured by 8-OHdG level) and oxidative protein damage (as measured by protein carbonyl level) were detected in aging *O. melastigma*, and the levels were generally lower in females than in males (Table 4.6). E2 was inversely correlated with 8-OHdG [Spearman correlation coefficients (r) = -0.436; $p \leq 0.05$] and protein carbonyl content [Spearman correlation coefficients (r) = -0.636; $p \leq 0.001$], respectively (Table 3.4). The findings are consistent with studies in mammals that females exhibited lower oxidative damage than males (Loft et al. 1992; Chen et al. 1999). E2 is proposed as the key factor enhancing cellular antioxidant defense (Vina and Borras 2010) by mediating

transcriptional up-regulation of MnSOD and GPx antioxidant enzymes (Borras et al. 2003; Vina et al. 2003, 2005; Vina and Borras 2010). The antioxidant enzymes systems and regulation of antioxidant enzymes are highly conserved in vertebrates from fish to mammals (Landis and Tower 2005; Van Tiem and Di Giulio 2011). Conceivably, lower oxidative damage (DNA and protein) in the liver of female *O. melastigma* could be attributed to E2 up-regulation of MnSOD and GPx antioxidant enzymes. The present findings also show that gender difference in liver protein carbonyl content occurred earlier than that of 8-OHdG in *O. melastigma*, suggesting proteins are more susceptible to oxidative damage than DNA in the liver. The possible mechanisms of E2 mediated telomere maintenance in the liver of *O. melastigma* are summarized in Fig 4.6 (green box).

4.5.2 Possible mechanism(s) in the gills

Table 4.7 summarizes gender difference in plasma E2 level and gill TL, TA, TERT and ERα mRNA expressions, and 8-OHdG and protein carbonyl levels in *O. melastigma* at 4 months to 12 months old.

Table 4.7 Gender difference in plasma E2 level telomere length (TL), telomerase activity (TA), TERT and ERα mRNA expressions, and 8-OHdG and protein carbonyl levels in the gills of *O. melastigma* at 4 months to 12 months old.

Gill	Age		
	4 months	8 months	12 months
Plasma E2 level	F > M ***	F > M ***	n.s.
TL (< 2 kb)	F < M **	F < M **	n.s.
TA	F > M *	F > M ***	n.s.
TERT mRNA expression	n.s.	n.s.	n.s.
ERα mRNA expression	n.s.	n.s.	n.s.
8-OHdG	n.s.	F < M *	n.s.
Protein carbonyl	n.s.	F < M **	n.s.

*, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$; n.s. = no significant difference

Gender difference in TA was also detected in the gills of female medaka, except at very old age (12 months). This is, however, unlikely to be mediated via TERT transcription because no significant gender difference in TERT mRNA expression was found in *O. melastigma*. Given that a significant positive correlation was demonstrated between plasma E2 and gill TA [Spearman correlation coefficients (r) = 0.383; $p \leq 0.001$] (Table 3.2), the activity of telomerase enzyme in the gills of *O. melastigma* was likely to be induced by E2 via the TERT protein phosphorylation pathways, i.e. through PI3K/Akt signaling and/or PI3K/Akt/NF_KB signaling (Kang et al. 1999; Breitschopf et al. 2001; Kawagoe et al. 2003; Du et al. 2004; Kimura et al.

2004; Imanishi et al. 2005). ERα was found to be essential for interaction between E2 and PI3K. However, the undetectable or very low level of ERα mRNA expression in the gills of *O. melastigma* (Fig 3.10), which agrees with results obtained previously for channel fish, Atlantic salmon, seabream, eelpout and fathead minnow (Xia et al. 1999; Rogers et al. 2000; Socorro et al. 2000; Andreassen et al. 2003; Filby and Tyler 2005), may suggest that ERα is not involved in the increase in TA in the gills of female *O. melastigma*. Given the existence of multiple isoforms of ERs or ERRs (estrogen-related receptors) in *O. melastigma* tissues (Au et al, unpublished data), other ER isoform(s) (e.g. ERβ), rather than ERα, may be involved in the activation of PI3K. Taken together, a higher TA in the gills of female *O. melastigma* as compared to males may be mediated by TERT protein phosphorylation which involves the interaction of E2 with ER isoforms other than ERα (Fig 4.7, blue box).

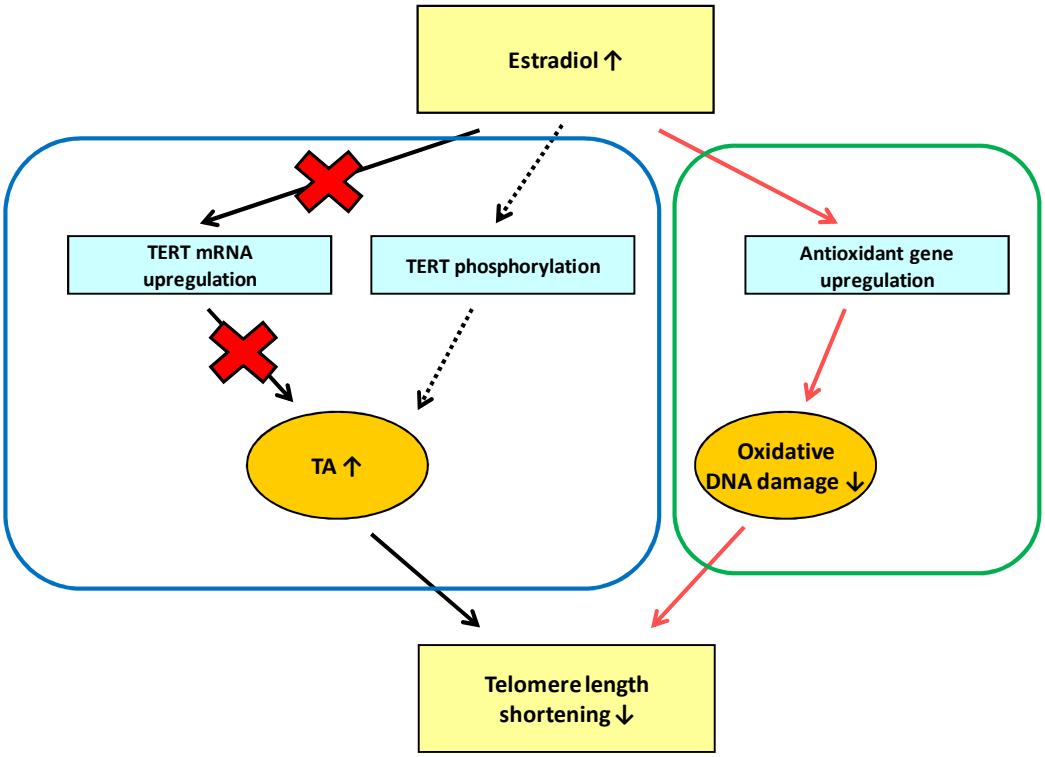


Fig 4.7 Possible mechanism of E2 up-regulation of telomerase activity (TA) and oxidative damage in the gills of *Oryzias melastigma*.

The gill is the most sensitive organ to oxidative stress (Sayeed et al. 2003) and has lower antioxidant potential than the liver (Sayeed et al. 2003; Oberdorster 2004; Monteiro et al. 2006). In *O. melastigma*, the 8-OHdG and protein carbonyl levels were lower in the gills of females than males at 8 months old, while no gender difference was detected in 4-month and 12-month old medaka (Table 4.7). It is possible that 8-OHdG and protein carbonyl accumulate more rapidly in middle-aged male *O. melastimga* (8 months) than in their female counterparts, since the females have stronger antioxidant defense capacity. When plasma E2 declined in old female *O. melastigma* (12 months), the antioxidant defense capacity in the female gills was

weakened and the gender advantage faded out dramatically. E2 was negatively correlated with 8-OHdG [Spearman correlation coefficients (r) = -0.542; $p \leq 0.01$], but no significant correlation was identified between E2 and protein carbonyl content ($p > 0.05$) (Table 3.4), indicating E2 is related to the reduction of 8-OHdG level in the gills. However, why E2 is not correlated with the reduction of protein carbonyl level in the gills remains obscure. The possible mechanisms for E2 mediated telomere maintenance in the gills of *O. melastigma* are summarized in Fig 4.7 (green box).

4.6 Conclusions

The present study confirms the existence of a longevity gender gap (LGG) in *O. melastigma*. E2, but not sexual size dimorphism, is a possible explanation for females living longer than males in *O. melastigma*. Gender differences in plasma E2 (F > M), telomerase activity (F > M) and oxidative damage (F < M) in the liver and gills were found in *O. melastigma*. The increase in TA is essential in replenishing shortened telomeres and slowing down telomere attrition. In the liver, E2 prevents telomere shortening by up-regulation of TERT gene and telomerase activity, although other mechanisms, such as the activation of telomerase by TERT protein phosphorylation, cannot be excluded. In the gills, E2 was likely to reduce telomere attrition through TERT protein phosphorylation, but unlikely by transactivation of TERT gene. The

findings of this study demonstrate tissue specific pathways in activation of TA by E2. Moreover, lower oxidative damage (DNA and protein) in tissues (liver and gills) of female *O. melastigma* may contribute to a longer lifespan in females. Both the induction of TA and the suppression of oxidative damage in females are responsible for gender specific telomere attrition in *O. melastigma* (male > female). Taken together, the findings in the present study support the view that E2 is a key factor contributing to the LGG in *O. melastigma*.

4.7 Future studies

(i) The present work studied only the pathway related to E2 activation of TA through up-regulation of TERT gene transcription in the liver and gills. The involvement of post-translational phosphorylation of TERT protein is largely speculative. Therefore, investigation of TERT phosphorylation by E2 in the liver and gills of *O. melastigma* would be constructive.

(ii) Due to the availability of omER and omERR isoforms other than omERα (e.g. omERβ) in *O. melastigma*, it would be worthwhile to study the mechanism of TERT activation by various omER/omERR isoforms through the interaction with E2 using medaka cells *in vitro*.

(iii) The present study confirms gender differences in DNA oxidation (as measured

by 8-OHdG level) and protein oxidation (as measured by protein carbonyl level) in *O. melastigma*. However, the involvement of E2 in up-regulation of antioxidant enzymes (e.g. MnSOD and GPx) is still speculative. It would be instructive to investigate the associated changes in the key antioxidant enzyme mRNA and protein expressions and enzymes activities.

(iv) Using *O. melastigma* for studying *in vivo* aging, the mechanistic relationship of E2-mediated up-regulation of TERT, TA and TL maintenance can be further verified by the alteration of plasma E2 level. For instance, synthetic estrogen, e.g. 17α-ethinylestradiol (EE2), can be used to test if increased plasma E2 may lead to a longer lifespan in male *O. melastigma*. Similarly, antiestrogen, e.g. ICI 182780, can be used to test if reduced plasma E2 results in a shorter lifespan in female *O. melastigma*.

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Appendix 1

Table 1 Life expectancy of female/male at birth, their difference, sex ratio at various ages and population size of a country in 2010 obtained from The World Factbook, CIA, USA (https://www.cia.gov/library/publications/the-world-factbook/rankorder/2012rank.html)

COUNTRY	LIFE EXPECTANCY AT BIRTH OF FEMALE (YEARS)	LIFE EXPECTANCY AT BIRTH OF MALE (YEARS)	DIFFERENCE OF LIFE EXPECTANCY BETWEEN BOTH SEXES (YEARS)	AT BIRTH [MALE(S)/FEMALE]	UNDER 15 [MALE(S)/FEMALE]	15-64 [MALE(S)/FEMALE]	65 [MALE(S)/FEMALE]	POPULATION
Monaco	93.9	85.81	8.09	1.048	1.05	1	0.8	30,586
Macau	87.49	81.42	6.07	1.05	1.14	0.88	0.88	567,957
San Marino	85.68	80.45	5.23	1.086	1.14	0.94	0.8	31,477
Japan	85.66	78.87	6.79	1.056	1.06	1.02	0.74	126,804,433
Hong Kong	84.88	79.24	5.64	1.076	1.09	0.94	0.88	7,089,705
Singapore	84.87	79.45	5.42	1.077	1.08	0.95	0.81	4,701,069
Guernsey	84.87	79.43	5.44	1.049	1.03	0.98	0.77	64,775
Andorra	84.55	80.3	4.25	1.066	1.06	1.09	0.99	84,525
France	84.44	77.91	6.53	1.051	1.05	1	0.72	64,768,389
Spain	84.27	78.06	6.21	1.065	1.06	1.01	0.72	46,505,963

Table 1 (continued)

COUNTRY	LIFE EXPECTANCY AT BIRTH OF FEMALE (YEARS)	LIFE EXPECTANCY AT BIRTH OF MALE (YEARS)	DIFFERENCE OF LIFE EXPECTANCY BETWEEN BOTH SEXES (YEARS)	AT BIRTH [MALE(S)/FEMALE]	UNDER 15 [MALE(S)/FEMALE]	15-64 [MALE(S)/FEMALE]	65 [MALE(S)/FEMALE]	POPULATION
Australia	84.25	79.33	4.92	1.055	1.05	1.03	0.84	21,515,754
Canada	84	78.72	5.28	1.056	1.05	1.02	0.78	33,759,742
Switzerland	83.95	78.14	5.81	1.054	1.08	1.02	0.72	7,623,438
Bermuda	83.88	77.37	6.51	1.018	1.01	0.97	0.71	68,265
Jersey	83.83	78.88	4.95	1.06	1.08	1	0.8	93,363
Liechtenstein	83.66	76.73	6.93	1.006	0.98	0.97	0.77	35,002
Italy	83.46	77.39	6.07	1.066	1.06	1.03	0.72	58,090,681
Sweden	83.4	78.69	4.71	1.061	1.06	1.02	0.8	9,074,055
Anguilla	83.39	78.22	5.17	1.032	1.05	0.9	0.93	14,766
Cayman Islands	83.27	77.91	5.36	1.016	1.01	0.95	0.89	50,209

Table 1 (continued)

COUNTRY	LIFE EXPECTANCY AT BIRTH OF FEMALE (YEARS)	LIFE EXPECTANCY AT BIRTH OF MALE (YEARS)	DIFFERENCE OF LIFE EXPECTANCY BETWEEN BOTH SEXES (YEARS)	AT BIRTH [MALE(S)/FEMALE]	UNDER 15 [MALE(S)/FEMALE]	15-64 [MALE(S)/FEMALE]	65 [MALE(S)/FEMALE]	POPULATION
Israel	83.12	78.7	4.42	1.05	1.05	1.03	0.78	7,353,985
Iceland	83.04	78.63	4.41	1.04	1.03	1.02	0.83	308,910
Luxembourg	82.95	76.22	6.73	1.066	1.07	1.01	0.7	497,538
Norway	82.89	77.42	5.47	1.054	1.04	1.03	0.76	4,676,305
Finland	82.76	75.64	7.12	1.04	1.04	1.02	0.69	5,255,068
Austria	82.71	76.74	5.97	1.051	1.05	1.01	0.71	8,214,160
Belgium	82.68	76.21	6.47	1.045	1.04	1.02	0.71	10,423,493
Germany	82.57	76.41	6.16	1.055	1.05	1.04	0.72	82,282,988
Puerto Rico	82.57	75.15	7.42	1.05	1.04	0.93	0.75	3,978,702
New Zealand	82.53	78.52	4.01	1.048	1.05	1	0.84	4,252,277

Table 1 (continued)

COUNTRY	LIFE EXPECTANCY AT BIRTH OF FEMALE (YEARS)	LIFE EXPECTANCY AT BIRTH OF MALE (YEARS)	DIFFERENCE OF LIFE EXPECTANCY BETWEEN BOTH SEXES (YEARS)	AT BIRTH [MALE(S)/FEMALE]	UNDER 15 [MALE(S)/FEMALE]	15-64 [MALE(S)/FEMALE]	65 [MALE(S)/FEMALE]	POPULATION
Greece	82.52	77.24	5.28	1.064	1.06	1	0.78	10,749,943
Bosnia and Herzegovina	82.49	75.09	7.4	1.074	1.07	1.02	0.69	4,621,598
Ireland	82.41	77.86	4.55	1.057	1.07	1	0.81	4,622,917
Virgin Islands	82.41	76.14	6.27	1.059	1.04	0.87	0.83	109,750
Netherlands	82.3	76.94	5.36	1.052	1.05	1.02	0.76	16,783,092
Korea, South	82.28	75.56	6.72	1.07	1.1	1.04	0.67	48,636,068
Faroe Islands	82.21	77.13	5.08	1.069	1.07	1.15	0.9	49,057
Isle of Man	82.18	79.01	3.17	1.077	1.05	1.01	0.71	83,859
Saint Pierre and Miquelon	82.12	77.49	4.63	1.04	1.06	1.03	0.68	5,943

Table 1 (continued)

COUNTRY	LIFE EXPECTANCY AT BIRTH OF FEMALE (YEARS)	LIFE EXPECTANCY AT BIRTH OF MALE (YEARS)	DIFFERENCE OF LIFE EXPECTANCY BETWEEN BOTH SEXES (YEARS)	AT BIRTH [MALE(S)/FEMALE]	UNDER 15 [MALE(S)/FEMALE]	15-64 [MALE(S)/FEMALE]	65 [MALE(S)/FEMALE]	POPULATION
United Kingdom	82.11	77.84	4.27	1.052	1.05	1.03	0.76	62,348,447
Malta	81.97	77.34	4.63	1.058	1.05	1.03	0.77	406,771
Wallis and Futuna	81.96	75.85	6.11	1.057	1.1	1.01	0.85	15,343
Portugal	81.86	75.12	6.74	1.067	1.09	1	0.7	10,735,765
Saint Helena, Ascension, and Tristan da Cunha	81.67	75.68	5.99	1.049	1.04	1.04	0.94	7,670
Gibraltar	81.56	75.69	5.87	1.071	1.06	1.02	0.93	28,877
Jordan	81.28	78.64	2.64	1.06	1.06	1.03	0.97	6,407,085

Table 1 (continued)

COUNTRY	LIFE EXPECTANCY AT BIRTH OF FEMALE (YEARS)	LIFE EXPECTANCY AT BIRTH OF MALE (YEARS)	DIFFERENCE OF LIFE EXPECTANCY BETWEEN BOTH SEXES (YEARS)	AT BIRTH [MALE(S)/FEMALE]	UNDER 15 [MALE(S)/FEMALE]	15-64 [MALE(S)/FEMALE]	65 [MALE(S)/FEMALE]	POPULATION
Taiwan	81.2	75.34	5.86	1.086	1.08	1.02	0.92	23,024,956
Slovenia	81.03	73.45	7.58	1.066	1.06	1.02	0.65	2,003,136
Denmark	80.97	76.11	4.86	1.055	1.05	1.01	0.78	5,515,575
Chile	80.96	74.26	6.7	1.05	1.05	1	0.72	16,746,491
New Caledonia	80.86	72.46	8.4	1.05	1.04	1.01	0.86	252,352
United States	80.81	75.78	5.03	1.047	1.04	1	0.75	310,232,863
Georgia	80.64	73.61	7.03	1.12	1.15	0.93	0.66	4,600,825
Cyprus	80.57	74.88	5.69	1.05	1.06	1.08	0.77	1,102,677
Panama	80.5	74.85	5.65	1.045	1.04	1.02	0.87	3,410,676
Czech Republic	80.48	73.74	6.74	1.059	1.06	1.01	0.66	10,201,707

Table 1 (continued)

COUNTRY	LIFE EXPECTANCY AT BIRTH OF FEMALE (YEARS)	LIFE EXPECTANCY AT BIRTH OF MALE (YEARS)	DIFFERENCE OF LIFE EXPECTANCY BETWEEN BOTH SEXES (YEARS)	AT BIRTH [MALE(S)/FEMALE]	UNDER 15 [MALE(S)/FEMALE]	15-64 [MALE(S)/FEMALE]	65 [MALE(S)/FEMALE]	POPULATION
Lithuania	80.29	70.23	10.06	1.057	1.06	0.96	0.53	3,545,319
Costa Rica	80.28	74.93	5.35	1.05	1.05	1.01	0.86	4,516,220
Argentina	80.17	73.52	6.65	1.052	1.05	1	0.7	41,343,201
Albania	80.11	74.65	5.46	1.123	1.1	1.05	0.87	2,986,952
Poland	80.06	71.88	8.18	1.061	1.06	0.99	0.62	38,463,689
Cuba	80.05	75.36	4.69	1.06	1.06	1	0.83	11,477,459
Uruguay	79.92	73.3	6.62	1.037	1.03	0.99	0.67	3,510,386
Libya	79.88	75.18	4.7	1.05	1.04	1.06	0.96	6,461,454
Slovakia	79.74	71.7	8.04	1.051	1.05	0.99	0.6	5,470,306
Northern Mariana Islands	79.68	74.27	5.41	1.059	1.15	0.85	0.92	48,317

Table 1 (continued)

COUNTRY	LIFE EXPECTANCY AT BIRTH OF FEMALE (YEARS)	LIFE EXPECTANCY AT BIRTH OF MALE (YEARS)	DIFFERENCE OF LIFE EXPECTANCY BETWEEN BOTH SEXES (YEARS)	AT BIRTH [MALE(S)/FEMALE]	UNDER 15 [MALE(S)/FEMALE]	15-64 [MALE(S)/FEMALE]	65 [MALE(S)/FEMALE]	POPULATION
French Polynesia	79.5	74.44	5.06	1.05	1.04	1.07	1.02	291,000
Saint Lucia	79.48	73.97	5.51	1.055	1.05	0.94	0.82	160,922
Croatia	79.4	71.95	7.45	1.055	1.06	0.99	0.64	4,486,881
Dominican Republic	79.38	75.01	4.37	1.04	1.04	1.04	0.86	9,823,821
Mexico	79.22	73.45	5.77	1.05	1.04	0.94	0.82	112,468,855
Kuwait §	79.18	76.64	2.54	**1.041**	1.04	1.79	**1.65**	2,789,132
United Arab Emirates §	79.01	73.75	5.26	**1.05**	1.05	2.75	**1.8**	4,975,593
Morocco	78.9	72.63	6.27	1.05	1.03	0.96	0.83	31,627,428
Dominica	78.87	72.82	6.05	1.05	1.04	1.05	0.76	72,813

Note: § indicates the country where sex ratio at birth is smaller than / equal to that over 65 years old.

Table 1 (continued)

COUNTRY	LIFE EXPECTANCY AT BIRTH OF FEMALE (YEARS)	LIFE EXPECTANCY AT BIRTH OF MALE (YEARS)	DIFFERENCE OF LIFE EXPECTANCY BETWEEN BOTH SEXES (YEARS)	AT BIRTH [MALE(S)/FEMALE]	UNDER 15 [MALE(S)/FEMALE]	15-64 [MALE(S)/FEMALE]	65 [MALE(S)/FEMALE]	POPULATION
British Virgin Islands §	78.78	76.18	2.6	**1.045**	1.03	1.05	**1.07**	24,939
Estonia	78.76	67.74	11.02	1.063	1.06	0.91	0.49	1,291,170
Paraguay	78.71	73.39	5.32	1.05	1.03	1.01	0.86	6,375,830
Aruba	78.61	72.47	6.14	1.021	1.01	0.92	0.66	104,589
Ecuador	78.6	72.58	6.02	1.05	1.04	0.97	0.93	14,790,608
Hungary	78.55	70.8	7.75	1.057	1.06	0.98	0.57	9,992,339
Brunei	78.31	73.72	4.59	1.047	1.06	0.99	0.94	395,027
Seychelles	78.09	68.6	9.49	1.031	1.05	1.09	0.6	88,340
Turks and Caicos Islands	78.07	73.32	4.75	1.047	1.04	1.1	0.98	23,528

Note: § indicates the country where sex ratio at birth is smaller than / equal to that over 65 years old.

Table 1 (continued)

COUNTRY	LIFE EXPECTANCY AT BIRTH OF FEMALE (YEARS)	LIFE EXPECTANCY AT BIRTH OF MALE (YEARS)	DIFFERENCE OF LIFE EXPECTANCY BETWEEN BOTH SEXES (YEARS)	AT BIRTH [MALE(S)/FEMALE]	UNDER 15 [MALE(S)/FEMALE]	15-64 [MALE(S)/FEMALE]	65 [MALE(S)/FEMALE]	POPULATION
Bahrain §	78.01	72.87	5.14	**1.03**	1.02	1.33	**1.13**	738,004
Tunisia	77.94	74.17	3.77	1.073	1.07	1.01	0.86	10,589,025
Mauritius	77.89	70.77	7.12	1.05	1.04	0.99	0.67	1,294,104
Colombia	77.84	70.98	6.86	1.06	1.05	0.97	0.74	44,205,293
Latvia	77.84	67.27	10.57	1.054	1.05	0.95	0.48	2,217,969
Macedonia	77.64	72.4	5.24	1.077	1.08	1.02	0.76	2,072,086
Sri Lanka	77.47	73.22	4.25	1.044	1.04	0.96	0.86	21,513,990
Romania	77.42	70.26	7.16	1.06	1.05	0.99	0.69	21,959,278
Cook Islands	77.38	71.69	5.69	1.046	1.13	1.07	0.96	11,488
Antigua and Barbuda	77.35	73.27	4.08	1.05	1.03	0.87	0.76	86,754

Note: § indicates the country where sex ratio at birth is smaller than / equal to that over 65 years old.

Table 1 (continued)

COUNTRY	LIFE EXPECTANCY AT BIRTH OF FEMALE (YEARS)	LIFE EXPECTANCY AT BIRTH OF MALE (YEARS)	DIFFERENCE OF LIFE EXPECTANCY BETWEEN BOTH SEXES (YEARS)	AT BIRTH [MALE(S)/FEMALE]	UNDER 15 [MALE(S)/FEMALE]	15-64 [MALE(S)/FEMALE]	65 [MALE(S)/FEMALE]	POPULATION
Qatar §	77.33	73.78	3.55	**1.056**	1.06	2.44	**1.36**	840,926
Thailand	77.21	72.94	4.27	1.054	1.05	0.98	0.82	67,089,500
Bulgaria	77.17	69.74	7.43	1.06	1.05	0.97	0.68	7,148,785
Serbia	77.1	71.26	5.84	1.065	1.07	1	0.7	7,344,847
American Samoa	77.08	71.04	6.04	1.061	1.04	1.03	0.88	66,432
Armenia	77.07	69.33	7.74	1.133	1.15	0.88	0.62	2,966,802
Venezuela	77	70.69	6.31	1.05	1.03	0.97	0.79	27,223,228
Syria	76.96	72.1	4.86	1.06	1.05	1.03	0.86	22,198,110
Belarus	76.93	65.26	11.67	1.062	1.06	0.94	0.47	9,612,632
West Bank	76.92	72.76	4.16	1.06	1.05	1.05	0.71	2,514,845

Note: § indicates the country where sex ratio at birth is smaller than / equal to that over 65 years old.

Table 1 (continued)

COUNTRY	LIFE EXPECTANCY AT BIRTH OF FEMALE (YEARS)	LIFE EXPECTANCY AT BIRTH OF MALE (YEARS)	DIFFERENCE OF LIFE EXPECTANCY BETWEEN BOTH SEXES (YEARS)	AT BIRTH [MALE(S)/FEMALE]	UNDER 15 [MALE(S)/FEMALE]	15-64 [MALE(S)/FEMALE]	65 [MALE(S)/FEMALE]	POPULATION
Suriname	76.91	71.24	5.67	1.068	1.04	0.99	0.75	486,618
China	76.77	72.54	4.23	1.14	1.17	1.06	0.93	1,330,141,295
Saint Kitts and Nevis	76.75	72.03	4.72	1.02	0.99	1.03	0.78	49,898
Solomon Islands	76.63	71.37	5.26	1.05	1.04	1.02	0.89	559,198
El Salvador	76.62	69.91	6.71	1.05	1.05	0.89	0.81	6,052,064
Maldives	76.54	72	4.54	1.05	1.04	1.57	0.98	395,650
Malaysia	76.48	70.81	5.67	1.069	1.06	1.01	0.79	28,274,729
Barbados	76.42	71.88	4.54	1.012	1	0.97	0.64	285,653
Lebanon	76.36	73.28	3.08	1.05	1.05	0.95	0.87	4,125,247
Algeria	76.04	72.57	3.47	1.05	1.04	1.02	0.86	34,586,184

Table 1 (continued)

COUNTRY	LIFE EXPECTANCY AT BIRTH OF FEMALE (YEARS)	LIFE EXPECTANCY AT BIRTH OF MALE (YEARS)	DIFFERENCE OF LIFE EXPECTANCY BETWEEN BOTH SEXES (YEARS)	AT BIRTH [MALE(S)/FEMALE]	UNDER 15 [MALE(S)/FEMALE]	15-64 [MALE(S)/FEMALE]	65 [MALE(S)/FEMALE]	POPULATION
Brazil	76	68.7	7.3	1.05	1.04	0.98	0.73	201,103,330
Saudi Arabia	75.9	71.93	3.97	1.05	1.04	1.27	1.03	25,731,776
Oman §	75.88	72.15	3.73	**1.05**	1.05	1.34	**1.06**	2,967,717
Saint Vincent and the Grenadines	75.82	72.04	3.78	1.03	1.02	1.06	0.83	104,217
Grenada	75.55	70.27	5.28	1.097	1.05	1.04	0.82	107,818
Uzbekistan	75.44	69.22	6.22	1.06	1.05	0.99	0.75	27,865,738
Gaza Strip	75.4	72.05	3.35	1.06	1.06	1.05	0.68	1,604,238
Jamaica	75.25	71.8	3.45	1.05	1.03	0.97	0.81	2,847,232
Samoa	75.13	69.28	5.85	1.05	1.07	1.08	0.8	192,001
Egypt	75.1	69.82	5.28	1.05	1.05	1.03	0.83	80,471,869

Note: § indicates the country where sex ratio at birth is smaller than / equal to that over 65 years old.

Table 1 (continued)

COUNTRY	LIFE EXPECTANCY AT BIRTH OF FEMALE (YEARS)	LIFE EXPECTANCY AT BIRTH OF MALE (YEARS)	DIFFERENCE OF LIFE EXPECTANCY BETWEEN BOTH SEXES (YEARS)	AT BIRTH [MALE(S)/FEMALE]	UNDER 15 [MALE(S)/FEMALE]	15-64 [MALE(S)/FEMALE]	65 [MALE(S)/FEMALE]	POPULATION
Moldova	75	67.39	7.61	1.059	1.06	0.94	0.58	4,317,483
Palau	74.84	68.36	6.48	1.065	1.06	1.25	0.43	20,879
Ukraine	74.74	62.56	12.18	1.065	1.06	0.92	0.49	45,415,596
Vietnam	74.69	69.48	5.21	1.115	1.1	0.99	0.62	89,571,130
Philippines	74.45	68.45	6	1.05	1.04	1	0.76	99,900,177
Turkey	74.19	70.37	3.82	1.05	1.05	1.02	0.84	77,804,122
Nicaragua	74.05	69.61	4.44	1.05	1.04	1	0.78	5,995,928
Trinidad and Tobago	74.02	68.23	5.79	1.028	1.05	1.06	0.75	1,228,691
Kyrgyzstan	73.94	65.74	8.2	1.053	1.04	0.96	0.64	5,508,626
Kazakhstan	73.78	62.91	10.87	1.058	1.04	0.95	0.53	15,460,484

Table 1 (continued)

COUNTRY	LIFE EXPECTANCY AT BIRTH OF FEMALE (YEARS)	LIFE EXPECTANCY AT BIRTH OF MALE (YEARS)	DIFFERENCE OF LIFE EXPECTANCY BETWEEN BOTH SEXES (YEARS)	AT BIRTH [MALE(S)/FEMALE]	UNDER 15 [MALE(S)/FEMALE]	15-64 [MALE(S)/FEMALE]	65 [MALE(S)/FEMALE]	POPULATION
Tonga	73.73	68.46	5.27	1.05	1.04	0.99	0.7	122,580
Fiji	73.73	68.46	5.27	1.05	1.04	1	0.81	875,983
Indonesia	73.69	68.53	5.16	1.05	1.04	1.01	0.79	242,968,342
Marshall Islands	73.65	69.41	4.24	1.05	1.04	1.04	0.94	65,859
Greenland	73.43	68.05	5.38	1.053	1.03	1.15	1.05	57,637
Bahamas, The	73.27	68.48	4.79	1.03	1.03	0.97	0.62	310,426
Micronesia, Federated States of	73.24	69.32	3.92	1.05	1.03	0.99	0.74	107,154
Russia	73.17	59.54	13.63	1.06	1.06	0.92	0.44	139,390,205
Peru	73	69.14	3.86	1.046	1.04	1.01	0.89	29,907,003

Table 1 (continued)

COUNTRY	LIFE EXPECTANCY AT BIRTH OF FEMALE (YEARS)	LIFE EXPECTANCY AT BIRTH OF MALE (YEARS)	DIFFERENCE OF LIFE EXPECTANCY BETWEEN BOTH SEXES (YEARS)	AT BIRTH [MALE(S)/FEMALE]	UNDER 15 [MALE(S)/FEMALE]	15-64 [MALE(S)/FEMALE]	65 [MALE(S)/FEMALE]	POPULATION
Cape Verde	72.64	68.24	4.4	1.03	1.01	0.94	0.61	508,659
Guatemala	72.51	68.76	3.75	1.05	1.04	0.94	0.86	13,550,440
Honduras	72.28	68.82	3.46	1.05	1.04	1.01	0.81	7,989,415
Iraq	71.69	68.88	2.81	1.05	1.04	1.03	0.88	29,671,605
Azerbaijan	71.67	62.86	8.81	1.124	1.13	0.97	0.58	8,303,512
Bangladesh	71.3	67.64	3.66	1.04	1.01	0.89	0.93	156,118,464
Iran	71.29	68.32	2.97	1.05	1.05	1.02	0.91	76,923,300
Turkmenistan	71.29	65.25	6.04	1.05	1.02	0.98	0.77	4,940,916
Montserrat *§	**70.91**	**74.82**	**-3.91**	**1.033**	1.09	0.91	**2.03**	5,118
Guyana	70.74	62.93	7.81	1.05	1.04	1	0.71	748,486

Notes: * indicates the country where life expectancy at birth of female is smaller than that of male.

§ indicates the country where sex ratio at birth is smaller than / equal to that over 65 years old.

Table 1 (continued)

COUNTRY	LIFE EXPECTANCY AT BIRTH OF FEMALE (YEARS)	LIFE EXPECTANCY AT BIRTH OF MALE (YEARS)	DIFFERENCE OF LIFE EXPECTANCY BETWEEN BOTH SEXES (YEARS)	AT BIRTH [MALE(S)/FEMALE]	UNDER 15 [MALE(S)/FEMALE]	15-64 [MALE(S)/FEMALE]	65 [MALE(S)/FEMALE]	POPULATION
Mongolia	70.54	65.54	5	1.05	1.04	1	0.77	3,086,918
Timor-Leste	70.11	65.23	4.88	1.05	1.03	1.04	0.9	1,154,625
Bolivia	70.07	64.52	5.55	1.05	1.04	0.96	0.79	9,947,418
Belize	70	66.54	3.46	1.05	1.04	1.02	0.91	314,522
Tajikistan	68.88	62.63	6.25	1.05	1.04	0.98	0.74	7,487,489
Nauru §	68.39	60.93	7.46	**0.838**	1.04	0.97	**1**	9,267
Papua New Guinea §	68.31	63.78	4.53	**1.05**	1.03	1.06	**1.2**	6,064,515
Bhutan §	67.57	65.89	1.68	**1.05**	1.04	1.13	**1.12**	699,847
India	67.57	65.46	2.11	1.12	1.13	1.07	0.91	1,173,108,018
Pakistan	67.5	63.84	3.66	1.05	1.06	1.09	0.92	184,404,791

Note: § indicates the country where sex ratio at birth is smaller than / equal to that over 65 years old.

Table 1 (continued)

COUNTRY	LIFE EXPECTANCY AT BIRTH OF FEMALE (YEARS)	LIFE EXPECTANCY AT BIRTH OF MALE (YEARS)	DIFFERENCE OF LIFE EXPECTANCY BETWEEN BOTH SEXES (YEARS)	AT BIRTH [MALE(S)/FEMALE]	UNDER 15 [MALE(S)/FEMALE]	15-64 [MALE(S)/FEMALE]	65 [MALE(S)/FEMALE]	POPULATION
Nepal	67.05	64.62	2.43	1.04	1.04	0.92	0.88	28,951,852
Burma	66.94	62.23	4.71	1.06	1.04	0.99	0.77	53,414,374
Korea, North	66.89	61.53	5.36	1.06	1.03	0.98	0.64	22,757,275
Tuvalu	66.51	62.36	4.15	1.051	1.06	0.95	0.73	10,472
Kiribati	66.49	61.68	4.81	1.05	1.04	0.95	0.64	99,482
Comoros	66.32	61.41	4.91	1.03	1.01	0.98	0.83	773,407
Vanuatu §	66.04	62.7	3.34	**1.05**	1.04	1.04	**1.05**	221,552
Mayotte §	65.63	60.99	4.64	**1.03**	1.01	1.15	**1.05**	231,139
Yemen	65.47	61.35	4.12	1.05	1.04	1.03	0.91	23,495,361
Madagascar	65.3	61.27	4.03	1.03	1.01	0.99	0.8	21,281,844

Note: § indicates the country where sex ratio at birth is smaller than / equal to that over 65 years old.

Table 1 (continued)

COUNTRY	LIFE EXPECTANCY AT BIRTH OF FEMALE (YEARS)	LIFE EXPECTANCY AT BIRTH OF MALE (YEARS)	DIFFERENCE OF LIFE EXPECTANCY BETWEEN BOTH SEXES (YEARS)	AT BIRTH [MALE(S)/FEMALE]	UNDER 15 [MALE(S)/FEMALE]	15-64 [MALE(S)/FEMALE]	65 [MALE(S)/FEMALE]	POPULATION
Togo	64.83	59.74	5.09	1.03	1	0.96	0.63	6,587,239
Cambodia	64.72	59.95	4.77	1.045	1.02	0.95	0.6	14,453,680
Eritrea	64.3	60.06	4.24	1.03	1.01	0.96	0.82	5,792,984
Laos	63.94	60.14	3.8	1.04	1.01	0.98	0.75	6,368,162
Sao Tome and Principe	63.91	61.58	2.33	1.03	1.03	0.97	0.86	175,808
Haiti	63.53	60.84	2.69	1.011	1.02	0.99	0.62	9,719,932
Djibouti	63.22	58.31	4.91	1.03	1	0.8	0.81	740,528
Mauritania	62.99	58.57	4.42	1.03	1.01	0.89	0.74	3,205,060
Western Sahara	62.99	58.57	4.42	1.04	1.02	0.97	0.78	491,519

Table 1 (continued)

COUNTRY	LIFE EXPECTANCY AT BIRTH OF FEMALE (YEARS)	LIFE EXPECTANCY AT BIRTH OF MALE (YEARS)	DIFFERENCE OF LIFE EXPECTANCY BETWEEN BOTH SEXES (YEARS)	AT BIRTH [MALE(S)/FEMALE]	UNDER 15 [MALE(S)/FEMALE]	15-64 [MALE(S)/FEMALE]	65 [MALE(S)/FEMALE]	POPULATION
Equatorial Guinea	62.94	61.05	1.89	1.03	1.03	0.97	0.78	650,702
Ghana	61.78	59.36	2.42	1.03	1.02	1	0.84	24,339,838
Senegal	61.34	57.48	3.86	1.03	1.01	0.98	0.87	12,323,252
Botswana *	**60.75**	**61.11**	**-0.36**	1.03	1.04	1.02	0.68	2,029,307
Benin	60.68	58.21	2.47	1.05	1.04	0.99	0.69	9,056,010
Burundi	59.98	56.65	3.33	1.03	1.01	0.97	0.67	9,863,117
Kenya	59.32	58.33	0.99	1.02	1.01	1.01	0.83	40,046,566
Guinea	59.12	56.13	2.99	1.03	1.02	1	0.78	10,324,025
Rwanda	58.91	56.06	2.85	1.03	1.01	0.99	0.67	11,055,976
Ethiopia	58.39	53.28	5.11	1.03	1	0.96	0.75	88,013,491

Note: * indicates the country where life expectancy at birth of female is smaller than that of male.

Table 1 (continued)

COUNTRY	LIFE EXPECTANCY AT BIRTH OF FEMALE (YEARS)	LIFE EXPECTANCY AT BIRTH OF MALE (YEARS)	DIFFERENCE OF LIFE EXPECTANCY BETWEEN BOTH SEXES (YEARS)	AT BIRTH [MALE(S)/FEMALE]	UNDER 15 [MALE(S)/FEMALE]	15-64 [MALE(S)/FEMALE]	65 [MALE(S)/FEMALE]	POPULATION
Sierra Leone	58.18	53.27	4.91	1.03	0.98	0.92	0.81	5,245,695
Liberia §	58.14	55.05	3.09	**1.03**	1.01	0.98	**1.03**	3,685,076
Cote d'Ivoire	57.13	55.27	1.86	1.03	1.02	1.04	0.99	21,058,798
Congo, Democratic Republic of the	56.59	52.93	3.66	1.03	1.01	0.99	0.69	70,916,439
Gambia, The	55.86	52.32	3.54	1.03	1.01	0.98	0.98	1,824,158
Congo, Republic of the	55.84	53.27	2.57	1.03	1.01	0.99	0.7	4,125,916
Sudan §	55.44	53.04	2.4	**1.05**	1.04	1.01	**1.05**	43,939,598

Note: § indicates the country where sex ratio at birth is smaller than / equal to that over 65 years old.

Table 1 (continued)

COUNTRY	LIFE EXPECTANCY AT BIRTH OF FEMALE (YEARS)	LIFE EXPECTANCY AT BIRTH OF MALE (YEARS)	DIFFERENCE OF LIFE EXPECTANCY BETWEEN BOTH SEXES (YEARS)	AT BIRTH [MALE(S)/FEMALE]	UNDER 15 [MALE(S)/FEMALE]	15-64 [MALE(S)/FEMALE]	65 [MALE(S)/FEMALE]	POPULATION
Burkina Faso	55.31	51.39	3.92	1.03	1.01	1	0.64	16,241,811
Cameroon	54.9	53.21	1.69	1.03	1.02	1.01	0.85	19,294,149
Niger	54.26	51.75	2.51	1.03	1.02	0.99	0.8	15,878,271
Uganda	54.07	51.92	2.15	1.03	1.01	1.01	0.7	33,398,682
Tanzania	54.03	50.99	3.04	1.03	1.01	0.98	0.77	41,892,895
Mali	53.8	50.59	3.21	1.03	1.01	0.95	0.92	13,796,354
Gabon	53.58	51.96	1.62	1.03	1.01	1	0.72	1,545,255
Zambia	53.28	50.81	2.47	1.03	1.01	1.01	0.68	13,460,305
Somalia	51.94	48.12	3.82	1.03	1	1	0.7	10,112,453
Namibia *	**51.64**	**52.25**	**-0.61**	1.03	1.02	1.03	0.8	2,128,471

Note: * indicates the country where life expectancy at birth of female is smaller than that of male.

Table 1 (continued)

COUNTRY	LIFE EXPECTANCY AT BIRTH OF FEMALE (YEARS)	LIFE EXPECTANCY AT BIRTH OF MALE (YEARS)	DIFFERENCE OF LIFE EXPECTANCY BETWEEN BOTH SEXES (YEARS)	AT BIRTH [MALE(S)/FEMALE]	UNDER 15 [MALE(S)/FEMALE]	15-64 [MALE(S)/FEMALE]	65 [MALE(S)/FEMALE]	POPULATION
Malawi	51.64	50.22	1.42	1.015	1	1	0.76	15,447,500
Central African Republic	50.95	48.45	2.5	1.03	1.01	0.98	0.67	4,844,927
Lesotho	50.76	50.58	0.18	1.03	1.01	0.95	0.96	1,919,552
Guinea-Bissau	50.22	46.44	3.78	1.03	1	0.93	0.66	1,565,126
Chad	49.07	46.95	2.12	1.04	1.03	0.85	0.73	10,543,464
South Africa *	**48.29**	**50.08**	**-1.79**	1.02	1	1.02	0.68	49,109,107
Nigeria	48.08	46.46	1.62	1.06	1.05	1.04	0.94	152,217,341
Swaziland *	**47.8**	**48.14**	**-0.34**	1.03	1.02	0.98	0.72	1,354,051

Note: * indicates the country where life expectancy at birth of female is smaller than that of male.

Table 1 (continued)

COUNTRY	LIFE EXPECTANCY AT BIRTH OF FEMALE (YEARS)	LIFE EXPECTANCY AT BIRTH OF MALE (YEARS)	DIFFERENCE OF LIFE EXPECTANCY BETWEEN BOTH SEXES (YEARS)	AT BIRTH [MALE(S)/FEMALE]	UNDER 15 [MALE(S)/FEMALE]	15-64 [MALE(S)/FEMALE]	65 [MALE(S)/FEMALE]	POPULATION
Zimbabwe *	**47.11**	**47.98**	**-0.87**	1.03	1.02	0.83	0.75	11,651,858
Afghanistan	44.87	44.45	0.42	1.05	1.05	1.05	0.92	29,121,286
Mozambique *	**40.68**	**42.05**	**-1.37**	1.017	1.01	0.96	0.71	22,061,451
Angola	39.52	37.48	2.04	1.05	1.02	1.03	0.79	13,068,161

Note: * indicates the country where life expectancy at birth of female is smaller than that of male.

Appendix 2

Table 2 Life expectancy at birth by sex and their difference in Hong Kong, 1971-2010, obtained from The Demographic Statistics Section, Census and Statistics Department, Hong Kong Government (www.censtatd.gov.hk)

YEAR	LIFE EXPECTANCY AT BIRTH (YEARS)		DIFFERENCE (YEARS)
	FEMALE	MALE	
1971	75.3	67.8	7.5
1972	75.4	67.7	7.7
1973	75.9	68.5	7.4
1974	76.3	69.1	7.2
1975	76.8	70.1	6.7
1976	76.2	69.6	6.6
1977	76.7	70.1	6.6
1978	76.7	70.6	6.1
1979	76.9	70.6	6.3
1980	77.9	71.6	6.3
1981	78.5	72.3	6.2
1982	78.4	72.6	5.8
1983	78.4	72.3	6.1
1984	79	73.2	5.8
1985	79.2	73.8	5.4
1986	79.4	74.1	5.3
1987	79.7	74.2	5.5
1988	79.9	74.4	5.5
1989	80	74.2	5.8
1990	80.3	74.6	5.7
1991	80.7	75.2	5.5
1992	80.7	74.8	5.9
1993	80.9	75.3	5.6
1994	81.5	75.7	5.8
1995	81.5	76	5.5
1996	82.7	76.7	6
1997	83.2	77.2	6
1998	83	77.4	5.6
1999	83.2	77.7	5.5

Table 2 (continued)

YEAR	LIFE EXPECTANCY AT BIRTH (YEARS)		DIFFERENCE (YEARS)
	FEMALE	MALE	
2000	83.9	78	5.9
2001	84.6	78.4	6.2
2002	84.5	78.5	6
2003	84.4	78.5	5.9
2004	84.8	79	5.8
2005	84.6	78.8	5.8
2006	85.5	79.4	6.1
2007	85.5	79.4	6.1
2008	85.5	79.3	6.2
2009	85.9	79.7	6.2
2010	85.9 *	80 *	5.9 *

Notes: * indicates provisional figures.

Figures from 1971 to 1995 are complied based on “extended de facto” approach and those from 1996 onwards based on “resident population” approach.

Appendix 3

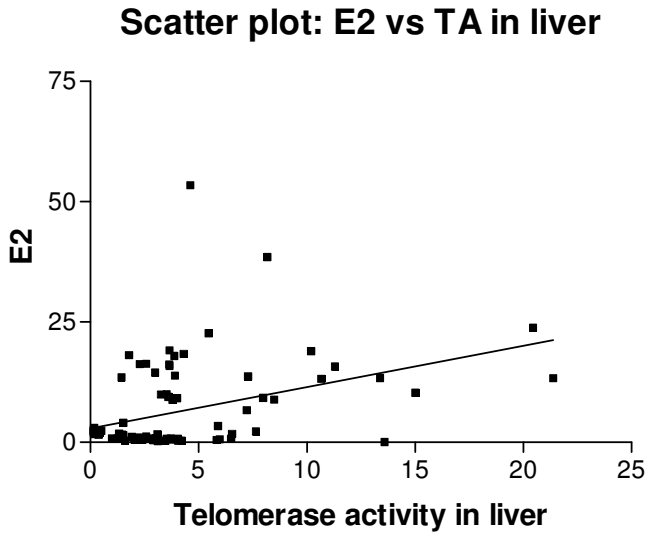

Fig 1 Spearman correlation between E2 and telomerase activity (TA) in the liver of *Oryzias melastigma* ($r = 0.224$; $p \leq 0.05$; $n = 90$).

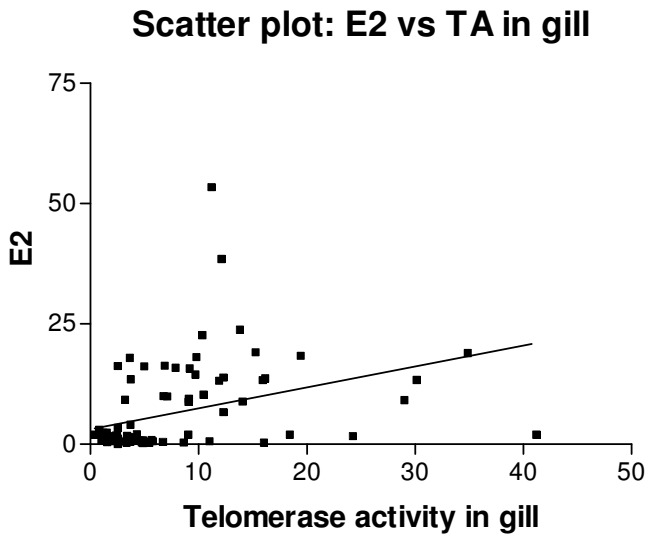

Fig 2 Spearman correlation between E2 and TA in the gills of *Oryzias melastigma* ($r = 0.383$; $p \leq 0.001$; $n = 90$).

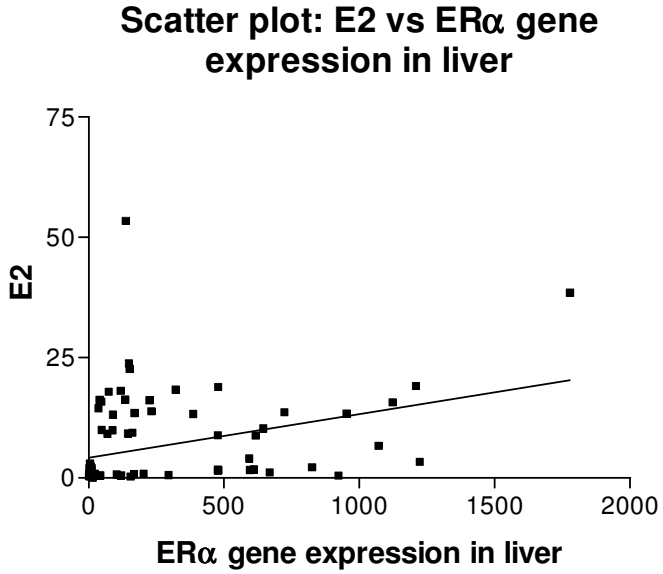

Fig 3 Spearman correlation between E2 and ERα gene expression in the liver of *Oryzias melastigma* (r = 0.471; p ≤ 0.001; n = 90).

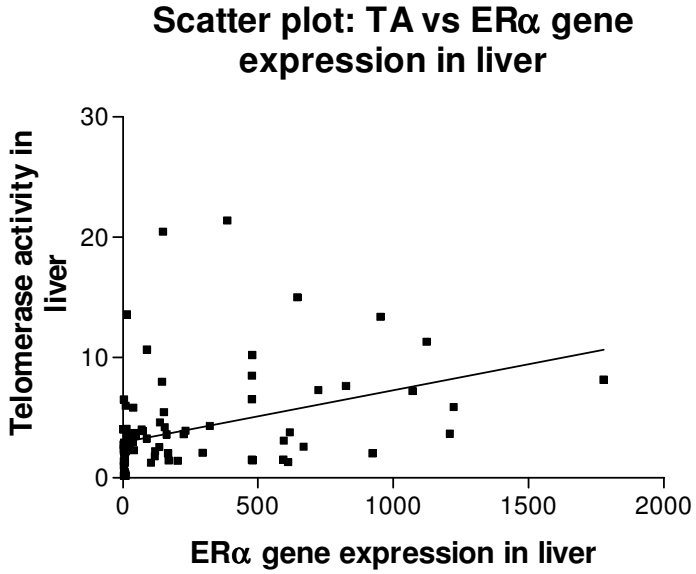

Fig 4 Spearman correlation between TA and ERα gene expression in the liver of *Oryzias melastigma* (r = 0.489; p ≤ 0.001; n = 90).

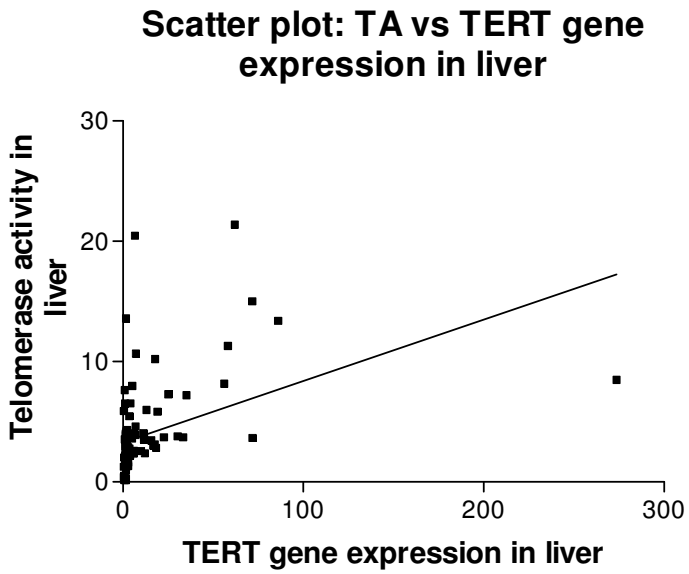

Fig 5 Spearman correlation between TA and TERT gene expression in the liver of *Oryzias melastigma* (r = 0.649; $p \leq 0.001$; n = 90).

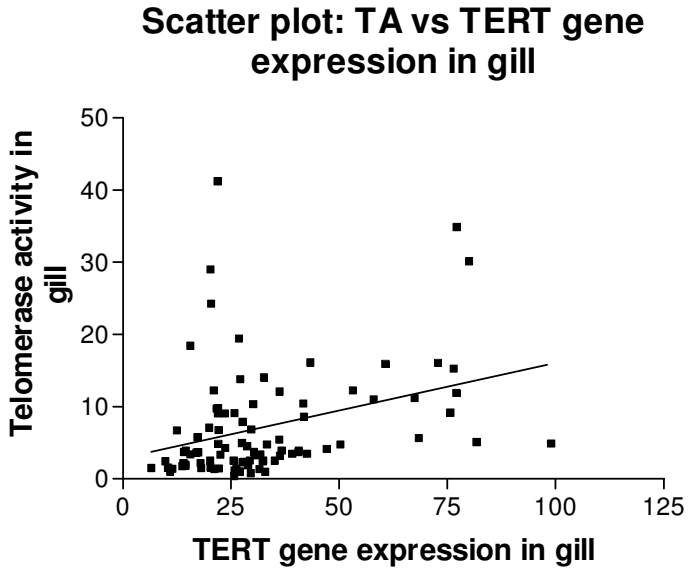

Fig 6 Spearman correlation between TA and TERT gene expression in the gills of *Oryzias melastigma* (r = 0.379; $p \leq 0.001$; n = 90).

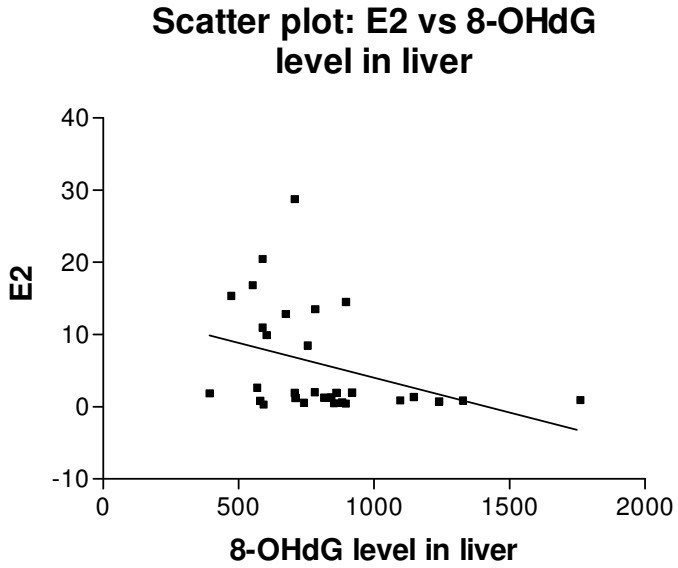

Fig 7 Spearman correlation between E2 and 8-OHdG level in the liver of *Oryzias melastigma* (r = -0.436; $p \leq 0.05$; n = 30).

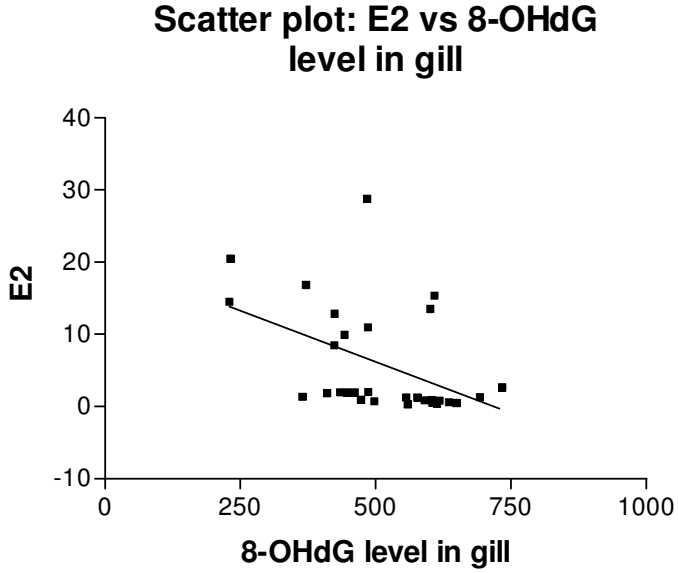

Fig 8 Spearman correlation between E2 and 8-OHdG level in the gills of *Oryzias melastigma* (r = -0.542; $p \leq 0.01$; n = 30).

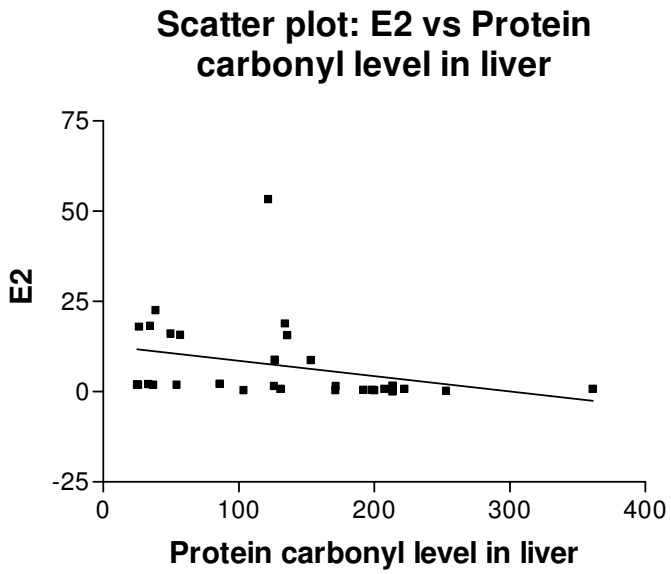

Fig 9 Spearman correlation between E2 and protein carbonyl level in the liver of *Oryzias melastigma* ($r = -0.636$; $p \leq 0.001$; $n = 30$).