The crustacean Armadillidium vulgare (Latreille, 1804) (Isopoda: Oniscoidea), a new promising model for the study of cellular senescence

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4 **The crustacean *Armadillidium vulgare* (Latreille, 1804) (Isopoda:**

5 **Oniscoidea)*,* a new promising model for the study of cellular**

6 **senescence**

7

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24

25   ABSTRACT

26   Senescence, the decline of physiological parameters with increasing age, is a quasi-ubiquitous

27   phenomenon in the living world. The observed patterns of senescence, however, can markedly

28   differ across species and populations, between sexes, and even among individuals. To identify

29   the drivers of this variation in senescence, experimental approaches are essential and involve

30   the development of tools and new study models. Current knowledge of the senescence process

31   is mostly based on studies on vertebrates and main information about senescence in

32   invertebrates is mostly limited to model organisms such as *Caenorhabditis elegans* or

33   *Drosophila melanogaster*. In this context, we tested whether biomarkers of vertebrate aging

34   could be used to study senescence in a new invertebrate model: the common woodlouse

35   *Armadillidium vulgare* (Latreille, 1804). More specifically, we looked for the effect of age in

36   woodlouse on three well-established physiological biomarkers of aging in vertebrates:

37   immune cells (cell size, density, and viability), β-galactosidase activity, and the gene

38   expression of telomerase reverse transcriptase (TERT), an essential subunit of telomerase

39   protein. We found that the size of immune cells was higher in older individuals, whereas their

40   density and viability decreased, and that the β-galactosidase activity increased with age,

41   whereas the TERT gene expression decreased. These findings demonstrate that woodlouse

42   display age-related changes in biomarkers of vertebrate senescence, with different patterns

43   depending on gender. The tools used in studies of vertebrate senescence can thus be

44   successfully used in studies of senescence of invertebrates such as the woodlouse. The

45   application of commonly used tools to new biological models offers a promising approach to

46   assess the diversity of senescence patterns across the tree of life.

47

48   **Key Words**: immunosenescence, telomerase reverse transcriptase (TERT), β-galactosidase

49   activity

50

51   INTRODUCTION

52   Many hypotheses have tried to explain why senescence is a quasi-ubiquitous phenomenon in

53   the living organisms. For instance, the disposable soma theory proposed the senescence

54   process as a result of damages accumulation over time (Kirkwood, 1977). These damages are

55   strongly influenced by the environment, leading to trade-offs between the different functions

56   (e.g., between reproduction and somatic maintenance) and shaping a high diversity of

57   senescence patterns across species and populations, among individuals, and between sexes.

58   One current challenge is to understand the selective forces and mechanisms driving this

59   diversity of senescence patterns.

60   At the cellular level, senescence corresponds to the cellular deterioration leading to

61   stop the cellular cycle (Campisi & d'Adda di Fagagna, 2007). As ageing is associated with

62   cellular senescence (Herbig *et al.*, 2006; Wang *et al.*, 2009; Lawless *et al.*, 2010), many

63   biomolecular parameters potentially inform about senescence and can therefore be valuable

64   tools for studying this process (Bernardez de Jesus & Blasco, 2012). For example, the

65   evolution of the integrity and efficiency of immune cells is particularly relevant to study

66   cellular senescence because a diminution of the number of effective immune cells with

67   increasing age takes place in both vertebrates (e.g., Cheynel *et al.*, 2017) and invertebrates

68   (e.g., Park *et al.*, 2011). Another marker used to study cellular senescence is the enzymatic

69   activity of the β-galactosidase. This enzyme is a hydrolase that transforms polysaccharides

70   into monosaccharides. The lysosomal activity of this enzyme is increased when the cell enters

71   in senescence (Dimri *et al.*, 1995; Itahana *et al.*, 2007). This phenomenon occurs in senescent

72   cells of many organisms ranging from humans (Gary & Kindell, 2005) to honeybees (Hsieh &

73   Hsu, 2011). Another protein linked to the cellular senescence process is the telomerase, a

74   ribonucleoprotein complex composed by two essential components, the telomerase reverse

75   transcriptase (TERT) and the telomerase RNA (TR) and other accessorial proteins (Podlevsky

76   *et al.*, 2007). Telomerase lengthens the ends of telomeres (i.e., DNA sequences located at the

77   end of chromosomes that protect chromosome integrity and shorten after each cell division).

78   Cell senescence arises when the telomere length becomes critically short (Chiu & Harley,

79   1997; Shay & Wright, 2005). Telomerase activity depends on the species, age, as well as the

80   type of tissue (e.g., Gomes *et al.*, 2010). For instance, telomerase is active during the

81   development before birth and after only in stem and germ cells in humans (Liu *et al.*, 2007;

82   Morgan, 2013) whereas in *Daphnia pulicaria* Forbes, 1893 the telomerase activity in all

83   tissues of the body decreases with increasing age (Schumpert *et al.*, 2015). The TERT is

84   essential in the telomerase protein complex and has been shown to be related to cell survival

85   in humans (Cao *et al.*, 2002). The TERT has also been detected in numerous species including

86   vertebrates, fungi, ciliates, and insects (Robertson & Gordon, 2006; Podlevsky *et al.*, 2007).

87   Because the patterns of senescence are strongly diversified within the living world, it

88   seems essential to study organisms displaying markedly different life histories strategies to

89   understand the causes and mechanisms underlying this diversity. Thus, invertebrates are

90   increasingly used in experimental studies of senescence (Stanley, 2012; Ram & Costa, 2018).

91   In addition to share similarities with vertebrates in terms of senescence, they can be

92   manipulated experimentally and they are easier to monitor throughout their entire lifetime

93   (Ram & Costa, 2018). These advantages make them models of choice for studying

94   senescence. Here, we propose the common woodlouse *Armadillidium vulgare* Latreille, 1804

95   as a promising new model for studying senescence. Woodlouse can live beyond three years

96   and display sex-specific senescence patterns in natural populations (Paris & Pitelka, 1962). In

97   addition, one study has already reported evidence of immune-senescence in this species

98   (Sicard *et al.*, 2010).

99   In this context, we tested the suitability of β-galactosidase activity, immune cell

100   parameters, and the TERT gene expression to cause age-specific responses in *A. vulgare.*

101   According to reports in the literature, we expected an increase in β-galactosidase activity, and

102   a decrease of both TERT gene expression and immune cell viability and density in *A. vulgare*.

103   As males have higher adult survival than females (Paris & Pitelka, 1962), cellular senescence

104   patterns are also expected to be sex-specific in this species.

105

MATERIALS AND METHODS

Biological model

Individuals of *A. vulgare* used in the experiments were obtained from a wild population

collected in Denmark in 1982. These individuals have been maintained on moistened soil

under the natural photoperiod of Poitiers, France (46.58°N, 0.34°E) at 20 °C, and fed *ad*

libitum with dried linden leaves and carrots. Crosses were monitored to control and promote

genetic diversity. For each clutch obtained, individuals were sexed, and brothers and sisters

were separated to ensure virginity. Woodlice are promiscuous and only breed when they are

one-year old during their first breeding season in the spring. Females carry their offspring in a

ventral pouch (marsupium) and can produce up to three clutches per season. In the common

woodlouse, individuals molt throughout their lifetimes, with approximate one molt per month.

During this process all the cells of the concerned tissues are renewed at 20 °C (Steel, 1980).

The brain, the nerve cord, and gonads, however, are not renewed during molting and are

therefore relevant candidates for tissue-specific study of senescence in this species. Woodlice

were classified in three different age categories: juvenile (before first reproduction, birth to

one-year old), young (one-year old, first year of reproduction), and old (up to two-year old),

which are very rare in natural populations (Paris & Pitelka, 1962). As the woodlouse is

continuous grower, juveniles and small individuals could not be used for certain experiments,

especially when they required protein extraction or hemolymph collection. Thus, we were

125   only able to test in them the telomerase expression. Old individuals were sampled according

126   to the number of individuals available in our breeding; sometimes we had two-year-old

127   individuals and sometimes three-year-old ones. Moreover, males and females were tested

128   separately to assess the impact of sex.

129

130   *Measurement of β-galactosidase activity*

131   To test the impact of age on β-galactosidase activity, 180 individuals were used: 90 young

132   (i.e., six-month old, 45 males, and 45 females) and 90 old (two-year old, 45 males, and 45

133   females).

134   Individuals were dissected separately in Ringer solution (sodium chloride (NaCl) 394

135   mM, potassium chloride (KCl) 2 mM, calcium chloride ($CaCl_2$) 2 mM, sodium bicarbonate

136   ($NaHCO_3$) 2 mM) and nerve cord was removed. To obtain a sufficient amount of protein, we

137   made pools of five nerve cords from five different individuals of the same age. The five nerve

138   cords were filed in 500 µl of Lyse buffer 1X (Chaps detergent ((3-((3-cholamidopropyl)

139   dimethylammonio)-1-propanesulfonate) 5 mM, citric acid ($C_6H_8O_7$) 40 mM, sodium

140   phosphate (Na_3PO_4) 40 mM, benzamidine ($C_7H_8N_2$) 0.5 mM, and PMSF

141   (phenymethylsulfonyle ($C_7H_7FO_2S$) 0.25 mM, pH 6) (Gary & Kindell, 2005), and then

142   centrifuged at 15,000g at 4 °C for 30 min. The supernatant was taken and kept at −80°C until

143   its utilization. The protein concentration was determined by the BCA (bicinchoninic acid

144   assay) assay (Thermo Fisher Scientific, Waltham, MA, USA) and homogenized at 0.1 mg ml

145 $^{-1}$. The β-galactosidase activity was measured as described by Gary & Kindell (2005). Briefly,

146 100 μl of extracted protein at the concentration of 0.1 mg ml $^{-1}$ were added to 100 μl of

147 reactive 4-methylumbelliferyl-D-galactopyranoside (MUG) solution in a 96-wellmicroplate.

148 The MUG reactive, in contact to β-galactosidase, leads by hydrolysis to the synthesis of 4-

149 methylumbelliferone (4-MU), which is detectable using fluorescent measurements. Measures

150 were performed by the multimode Mithrax microplate reader (LB940 HTS III, excitation

151 filter: 120 nm, emission filter 460 nm; Berthold Technologies, Bad Wildbad, Germany) for

152 120 min. Two technical replicates were measured for each nerve pool.

153

154 *Measurement of immune cell parameters*

155 To test the impact of age on the immune cell parameters (i.e., density, viability, and size), we

156 were able to undertake individual tests because of our previous experience. It was therefore

157 not necessary to carry out pools of hemolymph unlike proteins or DNA to make the

158 measurements. Sixty mature individuals were used: 30 young (i.e., one-year old, 15 males,

159 and 15 females) and 30 old (three-year old, 15 males, and 15 females) individuals.

160 To study the impact of age on the immune parameters, a hole was bored in the middle

161 of the sixth segment and 3 μl of haemolymph were collected per individual with an

162 eyedropper and deposited promptly in 15 μl of anticoagulant solution (MAS-EDTA (EDTA

163 (ethylenediaminetetraacetic ($C_{10}H_{16}N_2O_8$)) 9 mM, trisodium citrate ($Na_3C_6H_5O_7$) 27 mM,

164 sodium chloride (NaCl) 336 mM, glucose ($C_6H_{12}O_6$) 115 mM, pH 7 (Rodriguez *et al.*, 1995)).

165   Then, 6 µl of trypan blue at 0.4% (Invitrogen, Carlsbad, CA, USA) were added to color the

166   dead cells. Thereafter, 10 µl of this solution were deposed in a counting slide (Invitrogen

167   Coutness®; Thermo Fisher Scientific). The immune cell density, the immune cell viability,

168   and the immune cell size were evaluated using an automated cell counter (Invitrogen

169   Countess®).

170

171   *Measurement of TERT gene expression*

172   The identification of the telomerase reverse transcriptase (TERT) gene was first performed

173   from the *A. vulgare* genome (Chebbi *et al.*, 2019). In order to test whether this gene was

174   present and preserved in crustaceans, phylogenetic analyses were undertaken upstream (see

175   Supplementary material S1–S4). This gene has been found in crustacean transcriptomes and

176   the topology of the TERT-gene tree follows the phylogenetic relationships between the

177   involved species (Supplementary S3), suggesting a conserved role of the TERT gene.

178

179   *Gene expression*

180   We tested the effect of age on the expression of TERT gene within four different age groups:

181   1) four-month old, 2) one-year old, 3) two-year old, and 4) three-year old. Females and males

182   were tested separately by pools of five individuals in one-, two-, and three-year-old groups

183   and by pools of seven individuals in a four-month-old group as smaller individuals provide

184   less biological material. All conditions required four replicates for each sex. A total of 176

185 individuals were used for this experiment. For each group we tested the expression level of

186 the TERT gene in two different tissues: the nerve cord (somatic line) and gonads (germinal

187 line).

188 Animals were washed by immersion for 30 sec in a 30% sodium hypochlorite solution

189 (NaClO) followed by two 30-sec immersions in distilled water. Tissues were dissected in

190 Ringer solution (sodium chloride 394 mM, potassium chloride (KCl) 2 mM, calcium chloride

191 ($CaCl_2$) 2 mM, sodium bicarbonate ($NaHCO_3$) 2 mM) and deposited by pools of five units for

192 each tissues on TRIzol reagent (Invitrogen) to extract RNA according to the manufacturer's

193 protocol after a cell disintegration using a Vibra Cell 75,185 sonicator (amplitude of 30%).

194 Total RNA was quantified by NanoDrop technology and stored at –80 °C until use. Reverse

195 transcriptions (RT) were made from 500 ng of RNA previously extracted and using the

196 SuperScript^{TM} IV Reverse Transcriptase kit (Thermo Fisher Scientific) according to the

197 manufacturer's instructions. Primers were designed using the identified gene: primer

198 TERT_F: 5'-AGGGAAAACGATGCACAACC-3' and primer TERT_R: 5'-

199 GTTCGCCAAATGTTCGCAAC- 3' (see Supplementary material S1). Quantitative RT-PCR

200 was performed using 0.6 µl of each primer (10 µM), 2.4 µl of nuclease-free water, and 1.5 µl

201 of cDNA template and the LightCycler LC480 system (Roche, Pleasanton, CA, USA) as

202 follows: 10 min at 95 °C, 45 cycles of 10 s at 95 °C, 10 s at 60 °C, and 20 s at 72 °C.

203 Expression levels of target genes were normalized based on the expression level of two

204   reference genes previously established: the ribosomal protein L8 (RbL8) and the Elongation

205   Factor 2 (EF2) (Chevalier *et al.*, 2011).

206

207   *Statistical analyses.*

208   All statistical analyses were performed using the R software v.3.5.2 (R. Core Team, 2018).

209   The β-galactosidase activity was analyzed with linear mixed effect models using the package

210   lme4 (Bates *et al.*, 2014). As two technical replicates were measured for each pool, the model

211   included the pools fitted as a random effect, age and sex and their two-way interaction as fixed

212   factors.

213   Concerning the immune parameters, linear models with Gaussian distribution were

214   fitted to analyze variation in the cell size and viability. A linear model of the cell number (log-

215   transformed; (Ives, 2015) was fitted for the cell density.

216   The level of TERT expression according to age in the two different tissues were

217   compared by a Kruskal-Wallis rank sum test in combination with Nemenyi's post hoc

218   multiple comparison test with the Tuckey correction using R package PMCMR (Pohlert,

219   2014).

220   RESULTS

221   *β-galactosidase activity*

222   The β-galactosidase activity was higher in old (i.e., two-year old) than in young (i.e., six-

223   month old) individuals ($\chi^2_1 = 6.15$, $P = 0.013$; Fig. 1). We also detected a higher β-

224   galactosidase activity in females than in males ($\chi^2_1 = 7.26$, $P = 0.007$; Fig. 1).

225   *‹Fig. 1›*

226   *Immune cells parameters*

227   Cell size was higher in three-year-old than in one-year-old individuals ($F_{1,58} = 8.54$, $P =$

228   0.005; Fig. 2A). Conversely, the cell density was higher in one-year-old than in three-year-old

229   individuals ($F_{1,58} = 4.33$, $P = 0.01$; Fig. 2B). Concerning the immune cell viability, a

230   statistically significant interaction occurred between age and sex, with a relatively lower

231   immune cell viability in three-year-old females ($F_{3,56} = 6.85$, $P = 0.01$; Fig. 2C). No sex effect

232   was detected on cell size ($F_{2,57} = 0.76$, $P = 0.38$; Fig. 2A) or cell density ($F_{2,57} = 0.32$, $P =$

233   0.57, Fig. 2B).

234   *‹Fig. 2›*

235   *TERT gene expression*

236   The TERT gene expression decreased with increasing age in nerve cords ($\chi^2_3 = 23.30$, $P <$

237   0.001; Fig. 3A). TERT expression was higher in four-month-old individuals compared to two-

238   year-old and three-year-old individuals ($P = 0.001$ in both cases) and in one-year-old

239   individuals compared to three-years-old individuals ($P = 0.038$), without any detectable sex

240   effect ($\chi^2_1 = 0.14$, $P = 0.70$; Fig. 3A). In gonads, the TERT gene expression was much higher

241   in females ($\chi^2_1 = 17.81$, $P < 0.001$; Fig. 3B) and tended to decrease with increasing age ($\chi^2_3 =$

242   7.5, $P = 0.057$; Fig., 3B) as the TERT gene expression tended to be higher in four-month-old

243   females compared to three-year-old females ($P = 0.054$). A general tendency was also

244   observed in males ($\chi^2_1 = 7.34$, $P = 0.061$; Fig. 3B), the TERT gene expression tending to be

245   higher in two-year-old individuals compared to one-year-old and three-year-old individuals (P

246   = 0.14 and P = 0.12, respectively; Fig. 3B).

247   *<Fig. 3>*

248   DISCUSSION

249   We tested several effective physiological biomarkers of vertebrate senescence to assess

250   whether they could also be used in invertebrates such as the common woodlouse. Immune

251   cells showed an increase in their size and a decrease in their density and viability with

252   increasing age. The activity of the β-galactosidase enzyme in nerve cords increased. The

253   TERT gene expression decreased with increasing age in nerve cords of males and females and

254   in the gonads of females. In contrast, the TERT gene expression was very low in the male

255   gonads to suggest a role on the cellular senescence status in this tissue. The difference

256   regarding the expression of TERT in the nerve cords and gonads underlies the importance of

257   organ choice to perform such an analysis.

258   Our study is in line with previous studies that had previously revealed the possibility

259   of using vertebrate biomarkers in invertebrates (Hsieh & Hsu, 2011; Park *et al.*, 2011;

260   Schumpert *et al.*, 2015). By testing a set of different physiological biomarkers of vertebrate

261   senescence, often studied independently, our study supports both ideas that routinely used

262   biomarkers in vertebrates can be adapted in invertebrates and that the senescence process is

263   quasi-ubiquitous in the living world and can be expressed in a similar way in very different

264   organisms.

265   Previous studies have shown that the probabilities to survive decrease with increasing

266   age in *A. vulgare* (Paris & Pitelka, 1962). The cellular damages accumulated during the life of

267   the isopod could be the cause of cell senescence and therefore the driving force behind

268   actuarial senescence (Harman, 1956; Barja, 2000; Barja & Herrero, 2000; Finkel & Holbrook,

269   2000). In *A. vulgare*, the 2- and 3-year-old individuals could have therefore accumulated more

270   cellular damages during their lifetime, leading to the cellular senescence we report.

271   Our study also revealed a strong difference between sexes on the response of

272   biomarkers to age changes. At a given age, females display higher β-galactosidase activity

273   and lower immune cell viability than males, as if they age faster than males. Between-sex

274   differences in lifespan have been reported in *A. vulgare* with a longer lifespan in males than in

275   females (Geiser, 1934; Paris & Pitelka, 1962). Exact differences in actuarial senescence

276   patterns (i.e., age-specific changes in survival probabilities) remain to be quantified in *A.*

277   *vulgare* but such differences are quite common both in vertebrates and invertebrates (Tidière

278   *et al.*, 2015; Marais *et al.*, 2018). One of the main hypotheses proposed to explain sex

279   differences in longevity or senescence patterns relies on different resource allocation

280   strategies between sexes (Vinogradov, 1998; Bonduriansky *et al.*, 2008). Females carry their

281   offspring in their marsupium for one month, giving nutrients and protection, thus, they

282   allocate more energy to reproduction than males that do not provide any parental care. The

283   fact that the lifespan of *A. vulgare* is shorter in females (Paris & Pitelka, 1962) thus supports a

284   role of differential sex allocation in this species. Moreover, the difference between males in

285   TERT expression in gonads also suggest a difference in sex allocation according both to age

286   and sex. The increased in TERT expression in male gonad in two-years-old individuals should

287   be the result of a terminal investment in reproduction for males: by increasing their TERT

288   expression and thus potentially their telomerase activity they should provide a better fitness to

289   their offspring. This allocation may be lost when males are too old (three-year old).

290   Sex differences in resource allocation strategies could also be driven by environmental

291   conditions (Shertzer & Ellner, 2002). Our physiological biomarkers of vertebrate senescence

292   revealed sex differences, and as supported in Depeux *et al.* (2019), they could constitute

293   useful tools to identify other factors involved in variations in senescence patterns, such as

294   environmental stressors. Moreover, if these biomarkers seem to predict better the

295   physiological age than chronological age, notably in terms of survival and reproduction, they

296   could correspond to biomarkers of senescence in the woodlouse (Baker & Sprott, 1988; Simm

297   *et al.*, 2008; Sprott, 2010).

298   The physiological biomarkers of vertebrate senescence thus respond to age changes in

299   *A. vulgare*, a species that represents a new invertebrate model of aging. The parameters that

300   predict the chronological age of *A. vulgare* individuals might offer reliable biomarkers,

301   especially if their measurements are related to both reproductive and survival prospects more

302   than to the chronological age of individuals. The availability of its genome, the ease of its

303   breeding, its particularity to continue growing during its lifespan, and its adaptations to

304   terrestrial life as well as the presence of cellular senescence on our senescence biomarkers

305   make it a promising candidate to study senescence.

306

307   SUPPLEMENTARY MATERIAL

308   Supplementary material is available at *Journal of Crustacean Biology* online.

309   S1. Phylogenetic analysis protocol of the TERT gene in crustaceans.

310   S2. TERT-gene alignment used for the phylogenetic analysis.

311   S3. TERT-gene tree following the phylogenetic relationships between the species involved.

312   S4. TERT-gene tree following the phylogenetic relationships between the species involved

313   (Newick format).

314

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326

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443

444   **Figure captions**

445   **Figure 1**. Boxplot of β-galactosidase activity in *Armadillum vulgare* according to age and sex

446   (F, females, M, males). The thick line represents the median, the box the interquartile range,

447   and the whisker the most extreme data point within 1.5 of the interquartile range. The outliers

448   outside this range are shown as open circles. N = 24 pools of 5 individuals; * *P* < 0.05, **

449   P<0.01.

450

451   **Figure 1**. Immune cell size (**A**), density (**B**), and viability (**C**) according to age and sex in

452   *Armadillum vulgare* (F, females; M, males). The thick line represents the median, the box the

453   interquartile range, and the whisker the most extreme data point within 1.5 of the interquartile

454   range. The outliers outside this range are shown as open circles. N = 60 individuals: 15 one-

455   year-old females, 15 one-year-old males, 15 three-years-old females, and 15 three-years-old

456   males; * *P* < 0.05, ** *P* < 0.01.

457

458   **Figure 2**. Relative expression level of TERT expression in nerve cords (A) and in gonads (B)

459   in *Armadillum vulgare* (F, females; M, males). Expression of each gene was normalized based

460   on the expression of ribosomal protein L8 (RbL8) and elongation factor 2 (EF2) as reference

461   genes. The thick line represents the median, the box the interquartile range, and the whisker the

462   most extreme data point within 1.5 the interquartile range. N = 176 individuals: 28 four-month-

463   old females, 28 four-month-old males, 20 one-year-old females, 20 one-year-old males, 20 two-

464   year-old females, 20 two-year-old males, 20 three-year-old females, 20 three-year-old males; #

465   *P* < 0.10, ** *P* < 0.01

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