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

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ABSTRACT

Senescence, the decline of physiological parameters with increasing age, is a quasi-ubiquitous phenomenon in the living world. The observed patterns of senescence, however, can markedly differ across species and populations, between sexes, and even among individuals. To identify the drivers of this variation in senescence, experimental approaches are essential and involve the development of tools and new study models. Current knowledge of the senescence process is mostly based on studies on vertebrates and main information about senescence in invertebrates is mostly limited to model organisms such as *Caenorhabditis elegans* or *Drosophila melanogaster*. In this context, we tested whether biomarkers of vertebrate aging could be used to study senescence in a new invertebrate model: the common woodlouse *Armadillidium vulgare* (Latreille, 1804). More specifically, we looked for the effect of age in woodlouse on three well-established physiological biomarkers of aging in vertebrates: immune cells (cell size, density, and viability), β-galactosidase activity, and the gene expression of telomerase reverse transcriptase (TERT), an essential subunit of telomerase protein. We found that the size of immune cells was higher in older individuals, whereas their density and viability decreased, and that the β-galactosidase activity increased with age, whereas the TERT gene expression decreased. These findings demonstrate that woodlouse display age-related changes in biomarkers of vertebrate senescence, with different patterns depending on gender. The tools used in studies of vertebrate senescence can thus be successfully used in studies of senescence of invertebrates such as the woodlouse. The

application of commonly used tools to new biological models offers a promising approach to assess the diversity of senescence patterns across the tree of life.

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

## INTRODUCTION

Many hypotheses have tried to explain why senescence is a quasi-ubiquitous phenomenon in the living organisms. For instance, the disposable soma theory proposed the senescence process as a result of damages accumulation over time (Kirkwood, 1977). These damages are strongly influenced by the environment, leading to trade-offs between the different functions (e.g., between reproduction and somatic maintenance) and shaping a high diversity of senescence patterns across species and populations, among individuals, and between sexes. One current challenge is to understand the selective forces and mechanisms driving this diversity of senescence patterns.

At the cellular level, senescence corresponds to the cellular deterioration leading to stop the cellular cycle (Campisi & d'Adda di Fagagna, 2007). As ageing is associated with cellular senescence (Herbig *et al.*, 2006; Wang *et al.*, 2009; Lawless *et al.*, 2010), many biomolecular parameters potentially inform about senescence and can therefore be valuable 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

106 MATERIALS AND METHODS

107 *Biological model*

108 Individuals of *A. vulgare* used in the experiments were obtained from a wild population
109 collected in Denmark in 1982. These individuals have been maintained on moistened soil
110 under the natural photoperiod of Poitiers, France (46.58°N, 0.34°E) at 20 °C, and fed *ad*
111 *libitum* with dried linden leaves and carrots. Crosses were monitored to control and promote
112 genetic diversity. For each clutch obtained, individuals were sexed, and brothers and sisters
113 were separated to ensure virginity. Woodlice are promiscuous and only breed when they are
114 one-year old during their first breeding season in the spring. Females carry their offspring in a
115 ventral pouch (marsupium) and can produce up to three clutches per season. In the common
116 woodlouse, individuals molt throughout their lifetimes, with approximate one molt per month.
117 During this process all the cells of the concerned tissues are renewed at 20 °C (Steel, 1980).
118 The brain, the nerve cord, and gonads, however, are not renewed during molting and are
119 therefore relevant candidates for tissue-specific study of senescence in this species. Woodlice
120 were classified in three different age categories: juvenile (before first reproduction, birth to
121 one-year old), young (one-year old, first year of reproduction), and old (up to two-year old),
122 which are very rare in natural populations (Paris & Pitelka, 1962). As the woodlouse is
123 continuous grower, juveniles and small individuals could not be used for certain experiments,
124 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 ($C_6H_80_7$) 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 ($C_{10}H_{16}N_20_8$)) 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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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 $P < 0.10$, ** $P < 0.01$
466
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