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1 **The crustacean *Armadillidium vulgare*, a new**  
2 **promising model for the study of cellular senescence**

3

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17

18 Declarations of interest: none

19           **Abstract**

20           Senescence, the decline of physiological parameters with increasing age, is a quasi-  
21 ubiquitous phenomenon in the living world. However, the observed patterns of senescence  
22 can markedly differ between across species and populations, between sexes and even among  
23 individuals. To identify the drivers of this variation in senescence, experimental approaches  
24 are essential and involve the development of tools and new study models. In fact, current  
25 knowledge of the senescence process is mostly based on studies on vertebrates and principal  
26 information about senescence in invertebrates is mostly limited to model organisms such as  
27 *Caenorhabditis elegans* or *Drosophila melanogaster*. In this context, we tested whether  
28 biomarkers of vertebrate aging could be used to study senescence in a new invertebrate  
29 model: the common woodlouse *Armadillidium vulgare*. More specifically, we looked for the  
30 effect of age in woodlouse on three well established physiological biomarkers of aging in  
31 vertebrates: immune cells (cell size, density and viability),  $\beta$ -galactosidase activity, and  
32 Telomerase Reverse Transcriptase (TERT) (essential subunit of the telomerase protein) gene  
33 expression. We found that the size of immune cells was higher in older individuals, whereas  
34 their density and viability decreased, and that the  $\beta$ -galactosidase activity increased with age,  
35 whereas the Telomerase Reverse Transcriptase (TERT) gene expression decreased. These  
36 findings demonstrate that woodlouse display age-related changes in biomarkers of vertebrate  
37 senescence, with different patterns depending on gender. Thus, the tools used in studies of  
38 vertebrate senescence can be successfully used in studies of senescence of invertebrates such

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

41

## 42 **Keywords**

43 Cellular senescence, immunosenescence, Telomerase Reverse Transcriptase (TERT),  $\beta$ -  
44 galactosidase activity.

45

## 46 **1. Introduction**

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

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

59 studying this process (de Jesus & Blasco, 2012). For example, the evolution of the integrity  
60 and efficiency of immune cells is particularly relevant to study cellular senescence because a  
61 diminution of the number of effective immune cells with increasing age takes place in both  
62 vertebrates (e.g. Cheynel et al., 2017) and invertebrates (e.g. Park et al., 2011). Another  
63 marker used to study cellular senescence is the enzymatic activity of the  $\beta$ -galactosidase. This  
64 enzyme is a hydrolase that transforms polysaccharides in monosaccharides. The lysosomal  
65 activity of this enzyme is increased when the cell enters in senescence (Dimri *et al.*, 1995;  
66 Itahana *et al.*, 2007). This phenomenon occurs in senescent cells of many organisms ranging  
67 from humans (Gary & Kindell, 2005) to honeybees (Hsieh & Hsu, 2011). Another protein  
68 linked to the cellular senescence process is the telomerase, a ribonucleo protein complex  
69 composed by two essential components, the telomerase reverse transcriptase (TERT) and the  
70 telomerase RNA (TR) and other accessorial proteins (Podlevsky *et al.*, 2007). Telomerase  
71 lengthens the ends of telomeres (i.e. DNA sequences located at the end of chromosomes that  
72 protect chromosome integrity and shorten after each cell division). Cell senescence arises  
73 when the telomere length becomes critically short (Chiu & Harley, 1997; Shay & Wright,  
74 2005). The telomerase activity depends on organism, age and also tissues (e.g. (Gomes *et al.*,  
75 2010)). For instance, telomerase is active during the development before birth and after only  
76 in stem and germ cells in humans (Liu *et al.*, 2007; Morgan, 2013) while in the *Daphnia*  
77 *pulicaria*, the telomerase activity in all tissues of the body decreases with increasing age  
78 (Schumpert *et al.*, 2015). The TERT is essential in the telomerase protein complex and has

79 been shown to be related to cell survival in humans (Cao *et al.*, 2002). The TERT has also  
80 been detected in numerous species including vertebrates, fungi, ciliates and insects (Robertson  
81 & Gordon, 2006; Podlevsky *et al.*, 2007).

82 As patterns of senescence are strongly diversified within the living world, it seems  
83 essential to study organisms displaying markedly different life histories strategies to  
84 understand the causes and mechanisms underlying this diversity. Thus, invertebrates are  
85 increasingly used in experimental studies of senescence (Stanley, 2012; Ram & Costa, 2018).  
86 In addition to share similarities with vertebrates in terms of senescence, they can be  
87 manipulated experimentally and they are easier to be monitored throughout their entire  
88 lifetime (Ram & Costa, 2018). These advantages make them models of choice for studying  
89 senescence. Here, we propose the common woodlouse *A. vulgare* as a promising new model  
90 for studying senescence. Woodlouse can live beyond three years and display sex-specific  
91 senescence patterns in natural populations (Paris & Pitelka, 1962). In addition, one study has  
92 already reported evidence of immuno senescence in this species (Sicard *et al.*, 2010).

93

94 In this context, we tested the suitability of  $\beta$ -galactosidase activity, immune cell  
95 parameters and the TERT gene expression to cause age-specific responses in the common  
96 woodlouse *Armadillidium vulgare*. According to the literature, we expected an increase in  $\beta$ -  
97 galactosidase activity, and a decrease of both TERT gene expression and immune cell

98 viability and density in *A. vulgare*. As males have higher adult survival than females (Paris &  
99 Pitelka, 1962), cellular senescence patterns are also expected to be sex-specific in *A. vulgare*.

100

## 101 **2. Materials & Methods**

### 102 ***2.1. Biological model***

103 *A. vulgare* individuals used in the following experiments were derived from a wild  
104 population collected in Denmark in 1982. These animals have been maintained on moistened  
105 soil under the natural photoperiod of Poitiers (France 46.58°N, 0.34°E, 20°C) at 20°C fed *ad*  
106 *libitum* with dried linden leaves and carrots. Crosses were monitored to control and promote  
107 genetic diversity. For each clutch obtained, individuals were sexed, and brothers and sisters  
108 were separated to ensure virginity. In common woodlouse, individuals molt throughout their  
109 lives, with approximately one molt per month. During this process all the cells of the  
110 concerned tissues are renewed at 20°C (Steel, 1980). However, the brain, the nerve cord and  
111 gonads are not renewed during molting and are therefore relevant candidates for tissue-  
112 specific study of senescence in this species. Males and females were tested separately to  
113 assess the impact of sex.

114

### 115 ***2.2 Measure of $\beta$ -galactosidase activity***

#### 116 **Animals**

117 To test the impact of age on the on  $\beta$ -galactosidase activity, 180 individuals were used:  
118 90 young (i.e. 6-months-old, 45 males and 45 females) and 90 old (2-years-old, 45 males and  
119 45 females) individuals.

## 120 **Protocol**

121 Individuals were dissected separately in Ringer solution (Sodium Chloride 394 mM,  
122 Potassium Chloride 2 mM, Calcium Chloride 2 mM, Sodium Bicarbonate 2 mM) and nerve  
123 cord was removed. To obtain a sufficient amount of protein, we made pools of five nerve  
124 cords (from five different individuals of the same age). The five nerve cords were filed in 500  
125  $\mu$ L of Lyse Buffer 1X (CHAPS 5 mM, Citric acid 40 mM, Sodium Phosphate 40 mM,  
126 Benzamidine 0.5 mM, PMSF 0.25 mM, pH = 6) (Gary & Kindell, 2005), and then were  
127 centrifuged at 15000g at 4°C for 30 minutes. The supernatant was taken and kept at -80°C  
128 until its utilization. The protein concentration was determined by the BCA assay  
129 (Thermofisher) and was homogenized at 0.1 mg/mL. The  $\beta$ -galactosidase activity was  
130 measured as described by Gary and Kindell (2005). Briefly, 100  $\mu$ L of extracted protein at the  
131 concentration of 0.1 mg/mL were added to 100  $\mu$ L of reactive 4-methylumbelliferyl-D-  
132 galactopyranoside (MUG) solution in a 96 well-microplate. The MUG reactive, in contact to  
133  $\beta$ -galactosidase, leads by hydrolysis to the synthesis of 4-methylumbelliferone (4-MU), which  
134 is detectable using fluorescent measurements. Measures were performed by the multimode  
135 microplate reader Mithras (LB940 HTS III, Berthold; excitation filter: 120 nm, emission filter  
136 460 nm) for 120 minutes. Two technical replicates were measured for each pool.



137

## 138 ***2.3 Measure of immune cell parameters***

### 139 **Animals**

140 To test the impact of age on the immune cell parameters (i.e. density, viability, and  
141 size) in *A. vulgare*, 60 mature individuals were used: 30 young (i.e. 1-year-old, 15 males and  
142 15 females) and 30 old (3-years-old, 15 males and 15 females) individuals.

### 143 **Protocol**

144 To study the impact of age on the immune parameters, a hole was bored in the middle  
145 of the 6<sup>th</sup> segment and 3  $\mu$ L of haemolymph were collected (per individual) with an  
146 eyedropper and deposited promptly in 15  $\mu$ L of anticoagulant solution(MAS-EDTA (EDTA 9  
147 mM, Trisodium citrate 27 mM, NaCl 336 mM, Glucose 115 mM, pH 7, (Rodriguez *et al.*,  
148 1995))). Then, 6  $\mu$ L of Trypan blue at 0.4% (Invitrogen) were added to color the dead cells.  
149 Thereafter, 10  $\mu$ L of this solution were deposed in counting slide (Invitrogen Countess®,  
150 Thermofisher). The immune cell density, the immune cell viability and the immune cell size  
151 were evaluated using an automated Cell Counter (Invitrogen Countess®).

152

## 153 ***2.4 Measure of TERT gene expression***

154 The identification of the Telomerase Reverse Transcriptase (TERT)gene was firstly  
155 performed from the *A. vulgare* genome (Chebbi *et al.*, 2019). In order to check whether this  
156 gene was present and preserved in crustaceans, phylogenetic analyses were carried out

157 upstream (see Supplementary materials 1, 2, 3 and 4). This gene has been found in crustacean  
158 transcriptomes and the topology of the TERT gene tree follows the phylogenetic relationships  
159 between the involved species (Supplementary material 3), suggesting a conserved role of the  
160 TERT gene.

## 161 ***Gene expression***

### 162 **Animals**

163 We tested the effect of age on the expression of TERT gene within 4 different age  
164 groups: (1) 4-months-old, (2) 1-year-old, (3) 2-years-old and (4) 3-years-old. Females and  
165 males were tested separately by pools of 5 individuals in 1-, 2-, 3-years-old groups and by  
166 pools of 7 individuals in 4-months-old group. All conditions require 4 replicates for each sex.  
167 176 individuals were used for this experiment. For each group we tested the expression level  
168 of the TERT gene in two different tissues: the nerve cord (somatic line) and gonads (germinal  
169 line).

### 170 **Protocol**

171 Animals were washed by immersion for 30s in a 30% sodium hypochlorite solution  
172 followed by two 30s immersion in distilled water. Tissues were dissected in Ringer solution  
173 (Sodium Chloride 394 mM, Potassium Chloride 2 mM, Calcium Chloride 2 mM, Sodium  
174 Bicarbonate 2 mM) and deposited by specific tissues pools of 5 on TRIzol reagent  
175 (Invitrogen) to extract RNA according to the manufacturer's protocol after a cell  
176 disintegration using a Vibra Cell 75,185 sonicator (amplitude of 30%). Total RNA was

177 quantified by NanoDrop technology and was stored at -80°C until use. Reverse transcriptions  
178 (RT) were made from 500ng of RNA previously extracted and using the kit SuperScript™ IV  
179 Reverse Transcriptase (Thermo Fisher Scientific) according to the supplier's instructions.  
180 Primers were designed using the identified gene: primer TERT\_F: 5'-  
181 AGGGAAAACGATGCACAACC-3' and primer TERT\_R: 5'-  
182 GTTCGCCAAATGTTCGCAAC- 3' (see Supplementary material 1). Quantitative RT-PCR  
183 was performed using 0.6 µl of each primer (10 µM), 2.4 µl of nuclease-free water and 1.5 µl of  
184 cDNA template and the LightCycler LC480 system (Roche) with the following program: 10  
185 min at 95 °C, 45 cycles of 10 s at 95 °C, 10 s at 60 °C, and 20 s at 72 °C. Expression levels of  
186 target genes were normalized based on the expression level of two reference genes previously  
187 established: the Ribosomal Protein L8 (RbL8) and the Elongation Factor 2 (EF2) (Chevalier  
188 *et al.*, 2011).

189

## 190 **Statistics**

191 All statistical analyses were performed using the R software (R. Core Team,  
192 2016). The β-galactosidase activity was analyzed with linear mixed effect models using the  
193 package lme4 (Bates *et al.*, 2014). As two technical replicates were measured for each pool,  
194 the model including the pools fitted as a random effect, age and sex and their two-way  
195 interaction as fixed factors.

196 Concerning the immune parameters, linear models with Gaussian distribution were  
197 fitted to analyze variation in the cell size and viability. For the cell density, a linear model of  
198 the cell number (log-transformed, (Ives & Freckleton Robert, 2015)) was fitted.

199 The level of TERT expression according to age in the two different tissues were  
200 compared by a Kruskal–Wallis rank sum test in combination with Nemenyi’s post hoc  
201 multiple comparison test with the Tuckey correction using R package PMCMR.

202

### 203 **3. Results**

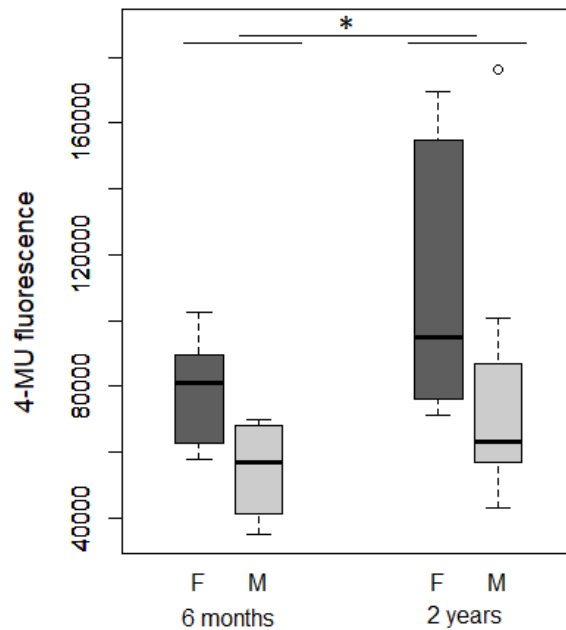
#### 204 **$\beta$ -galactosidase activity**

205 The  $\beta$ -galactosidase activity was higher in old (i.e. 2-years-old) than in young (i.e. 6-  
206 months-old) individuals ( $\chi^2_1=6.15$ ,  $p=0.013$ , Figure 1). We also detected a higher  $\beta$ -  
207 galactosidase activity in females than in males ( $\chi^2_1=7.26$ ,  $p=0.007$ , Figure 1).

208 The thick line depicts the median, the box the interquartile range, and the whisker are bounded to the most  
209 extreme data point within 1.5 the interquartile range. The outliers outside this range are displayed as open  
210 circles. N= 24 pools of 5 individuals. \* denotes  $p<0.05$

211

212



213

214 *Figure 1:  $\beta$ -galactosidase activity according to age and sex in A. vulgare (F=females, M=males)*

215

### 216 **Immune cells parameters**

217 Cell size was larger in 3-years-old than in 1-year-old individuals ( $F_{1,58}=8.54$ ,  $p=0.005$ , Figure

218 2A). Conversely, the cell density was higher in 1-year-old than in 3-years-old individuals

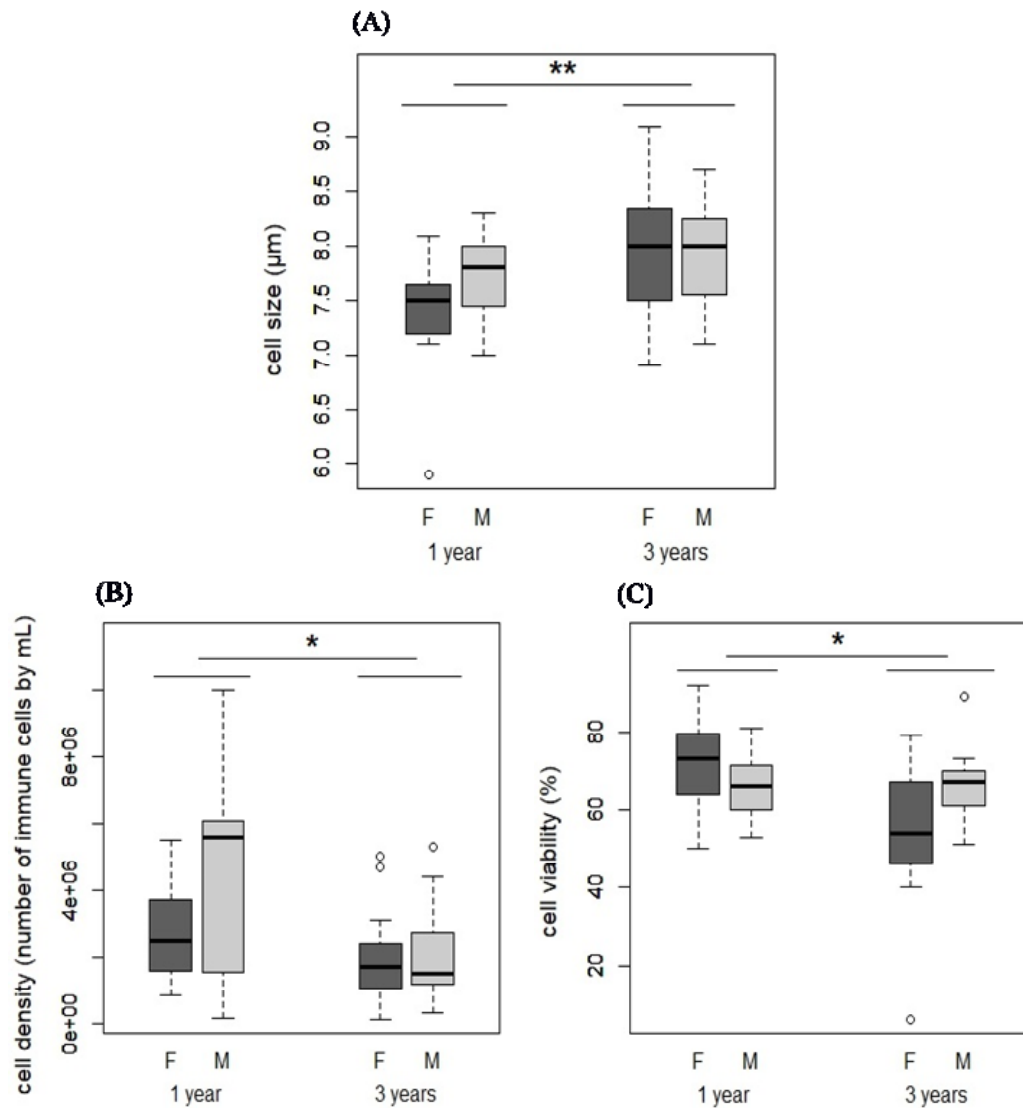
219 ( $F_{1,58} =4.33$ ,  $p=0.01$ , Figure 2B). Concerning the immune cell viability, a statistically

220 significant interaction occurred between age and sex, with a relatively lower immune cell

221 viability in 3-years-old females ( $F_{3,56}=6.85$ ,  $p=0.01$ , Figure 2C). No sex effect was detected on

222 cell size ( $F_{2,57}=0.76$ ,  $p=0.38$ , Figure 2A) or cell density ( $F_{2,57}=0.32$ ,  $p =0.57$ , Figure 2B).

223



224

225 **Figure 2: Immune cell size (A), density (B) and viability (C) according to age and sex in *A. vulgare***

226 (*F=females, M=males*)

227 The thick line depicts the median, the box the interquartile range, and the whisker are bounded to the most

228 extreme data point within 1.5 the interquartile range. The outliers outside this range are displayed as open

229 circles. N= 60 individuals: 15 1-year-old females, 15 1-year-old males, 15 3-years-old females and 15 3-years-old

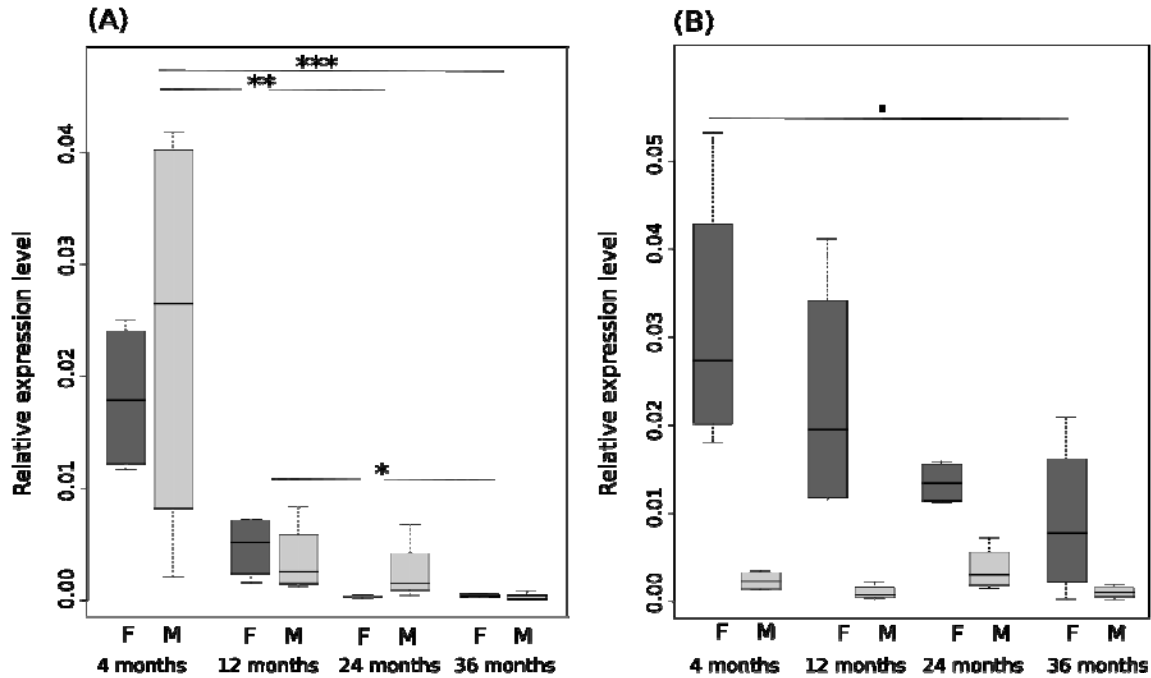
230 males. \* denotes  $p < 0.05$  and \*\* denotes  $p < 0.01$

231

232 **TERT gene expression**

233 The TERT gene expression decreased with increasing age in nerve cords ( $\chi^2_3=23.30$ ,  
234  $p<0.001$ , Figure 3A). More precisely, the TERT gene expression was higher in 4-months-old  
235 individuals compared to 2-years-old and 3-years-old individuals ( $p=0.001$  in both cases) and  
236 in 1-year-old individuals compared to 3-years-old individuals ( $p=0.038$ ), without any  
237 detectable sex effect ( $\chi^2_1=0.14$ ,  $p=0.70$ , Figure 3A). In gonads, the TERT gene expression  
238 was much higher in females ( $\chi^2_1=17.81$ ,  $p<0.001$ , Figure 3B) and tended to decrease with  
239 increasing age ( $\chi^2_3=7.5$ ,  $p=0.057$ , Figure 3B) as the TERT gene expression tended to be  
240 higher in 4-months-old females compared to 3-years-old females ( $p=0.054$ ). In males, a  
241 general tendency was also observed ( $\chi^2_1=7.34$ ,  $p=0.061$ , Figure 3B), the TERT gene  
242 expression tending to be higher in 2-years-old individuals compared to 1-year-old and 3-  
243 years-old individuals ( $p=0.14$  and  $p=0.12$ , respectively, Figure 3B).

244



245

246 *Figure 3: Relative expression level of TERT in (A) nerve cords and (B) in gonads in A. vulgare (F=females,*

247 *M=males.*

248 Expression of each gene was normalized based on the expression of Ribosomal Protein L8 (RbL8) and Elongation

249 Factor 2 (EF2) as reference genes. The thick line depicts the median, the box the interquartile range, and the whisker

250 are bounded to the most extreme data point within 1.5 the interquartile range. N= 176 individuals: 284-months-old

251 females, 28 4-months-old males, 20 1-year-old females, 20 1-year-old males, 20 2-years-old females, 20 2-years-old

252 males, 20 3-years-old females, 20 3-years-old males. . denotes  $p < 0.10$ , \*\* denotes  $p < 0.01$

253

## 254 4. Discussion

255 In this study, we tested several effective physiological biomarkers of vertebrate

256 senescence to assess whether they could also be used in invertebrates such as the common

257 woodlouse. Immune cells showed an increase in their size and a decrease in their density and



258 viability with increasing age. In nerve cords, the activity of the  $\beta$ -galactosidase enzyme  
259 increased, whereas the TERT gene expression decreased with increasing age. These results  
260 support the presence of increasing cellular senescence in *A. vulgare* with chronological age. In  
261 contrast, in the gonads, the TERT gene expression was too low in males and was not  
262 sufficiently variable between sexes to provide information on the cellular senescence status in  
263 this tissue.

264         Our study is in line with previous studies that have already revealed the possibility of  
265 using vertebrate biomarkers in invertebrates (Hsieh & Hsu, 2011; Park *et al.*, 2011;  
266 Schumpert *et al.*, 2015). By testing a set of different physiological biomarkers of vertebrate  
267 senescence, often studied independently, our study supports both ideas that routinely used  
268 biomarkers in vertebrates can be adapted in invertebrates and that the senescence process is  
269 quasi-ubiquitous in the living world and can be expressed in a similar way in very different  
270 organisms.

271         Previous studies have shown that the probabilities to survive decrease with increasing  
272 age in *A. vulgare* (Paris & Pitelka, 1962). The cellular damages accumulated during the  
273 animal's life could be the cause of cell senescence and therefore the driving force behind  
274 actuarial senescence. (Harman, 1956; Barja, 2000; Barja & Herrero, 2000; Finkel &  
275 Holbrook, 2000). In *A. vulgare*, the 2- and 3-years-old individuals could have therefore  
276 accumulated more cellular damages during their lifetime, leading to the cellular senescence  
277 we report.

278 Our study also revealed a strong difference between sexes on the response of  
279 biomarkers to age changes. At a given age, females display higher  $\beta$ -galactosidase activity  
280 and lower immune cell viability than males. Between-sex differences in lifespan have been  
281 reported in *A. vulgare* with a longer lifespan in males than in females (Geiser, 1934; Paris &  
282 Pitelka, 1962). Exact differences in actuarial senescence patterns (i.e. age-specific changes in  
283 survival probabilities) remain to be quantified in *A. vulgare* but such differences are quite  
284 common both in vertebrates and invertebrates (Tidière *et al.*, 2015; Marais *et al.*, 2018). One  
285 of the main theory proposed to explain sex differences in longevity or senescence patterns  
286 relies on different resource allocation strategies between sexes (Vinogradov, 1998;  
287 Bonduriansky *et al.*, 2008). The shorter lifespan in females *A. vulgare*, that allocate more  
288 energy to reproduction than males (Paris & Pitelka, 1962) because they carry their offspring  
289 in their marsupium during one month giving nutrients and protection, supports a role of  
290 differential sex allocation.

291 Sex differences in resource allocation strategies could also be driven by environmental  
292 conditions (Shertzer & Ellner, 2002). Our physiological biomarkers of vertebrate senescence  
293 revealed sex differences, and as supported in Depeux *et al.*, 2019, they could constitute useful  
294 tools to identify other factors involved in variations in senescence patterns, such as  
295 environmental stressors. Moreover, if these biomarkers seem to predict better the  
296 physiological age than chronological age notably in terms of survival and reproduction, they

297 could correspond to biomarkers of senescence in woodlouse (Baker & Sprott, 1988; Simm *et*  
298 *al.*, 2008; Sprott, 2010).

299 Our present study demonstrated that the physiological biomarkers of vertebrate  
300 senescence respond to age changes in the common woodlouse, a new invertebrate model of  
301 aging. These parameters that predict the chronological age of woodlouse individuals might  
302 offer reliable biomarkers, especially if their measurements are related to both reproductive  
303 and survival prospects more than to the chronological age of individuals. In this context, and  
304 more broadly in the study of senescence and of the factors involved in its diversity, the  
305 woodlouse model, which has physiological similarities with other invertebrates, could be a  
306 model of choice to study sex-specific actuarial and cellular senescence.

307

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313

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321

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