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

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Declarations of interest: none

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Abstract

Senescence, the decline of physiological parameters with increasing age, is a quasi-ubiquitous phenomenon in the living world. However, the observed patterns of senescence can markedly differ between 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. In fact, current knowledge of the senescence process is mostly based on studies on vertebrates and principal 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*. 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 Telomerase Reverse Transcriptase (TERT) (essential subunit of the telomerase protein) gene expression. 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 Telomerase Reverse Transcriptase (TERT) gene expression decreased. These findings demonstrate that woodlouse display age-related changes in biomarkers of vertebrate senescence, with different patterns depending on gender. Thus, the tools used in studies of vertebrate senescence can be successfully used in studies of senescence of invertebrates such

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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.

Keywords

Cellular senescence, immunosenescence, Telomerase Reverse Transcriptase (TERT), β-galactosidase activity.

1. Introduction

Many theories 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. 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 & 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

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

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

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

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

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

2. Materials & Methods

2.1. Biological model

A. vulgare individuals used in the following experiments were derived from a wild population collected in Denmark in 1982. These animals have been maintained on moistened soil under the natural photoperiod of Poitiers (France 46.58°N, 0.34°E, 20°C) at 20°C 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. In common woodlouse, individuals molt throughout their lives, with approximately one molt per month. During this process all the cells of the concerned tissues are renewed at 20°C (Steel, 1980). However, the brain, the nerve cord and gonads are not renewed during molting and are therefore relevant candidates for tissue-specific study of senescence in this species. Males and females were tested separately to assess the impact of sex.

2.2Measure of β-galactosidase activity

Animals

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

Protocol

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

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2.3Measure of immune cell parameters

Animals

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

Protocol

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

2.4 Measure of TERT gene expression

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

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

Gene expression

Animals

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

Protocol

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

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quantified by NanoDrop technology and was stored at -80°C until use. Reverse transcriptions (RT) were made from 500ng of RNA previously extracted and using the kit SuperScript™ IV Reverse Transcriptase (Thermo Fisher Scientific) according to the supplier's instructions. Primers were designed using the identified gene: primer TERT_F: 5'-AGGGAAAACGATGCACAACC-3' and primer TERT_R: 5'-GTTCGCCAAATGTTCGCAAC- 3' (see Supplementary material 1). Quantitative RT-PCR was performed using0.6 µl of each primer (10 µM), 2.4 µl of nuclease-free water and 1.5 µl of cDNA template and the LightCycler LC480 system (Roche) with the following program:10 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 target genes were normalized based on the expression level of two reference genes previously established: the Ribosomal Protein L8 (RbL8) and the Elongation Factor 2 (EF2) (Chevalier *et al.*, 2011).

Statistics

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

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

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

3. Results

β-galactosidase activity

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

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

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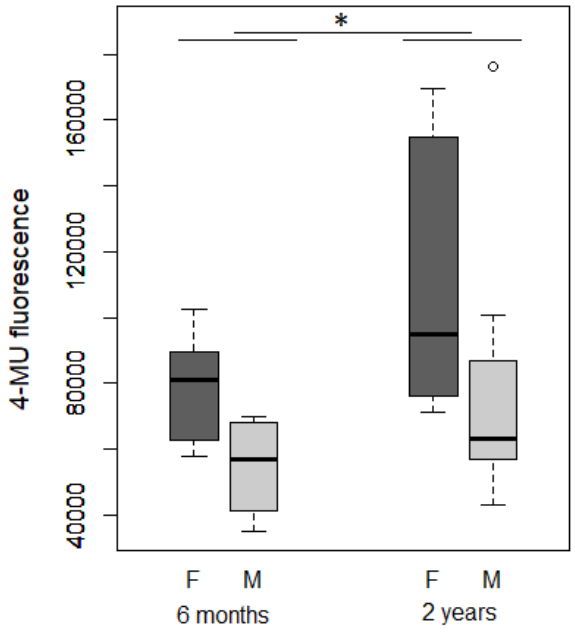

Figure 1: β-galactosidase activity according to age and sex in* A. vulgare *(F=females, M=males)

Immune cells parameters

Cell size was larger in 3-years-old than in 1-year-old individuals ($F_{1,58}$=8.54, p=0.005, Figure 2A). Conversely, the cell density was higher in 1-year-old than in 3-years-old individuals ($F_{1,58}$ =4.33, p=0.01, Figure 2B). Concerning the immune cell viability, a statistically significant interaction occurred between age and sex, with a relatively lower immune cell viability in 3-years-old females ($F_{3,56}$=6.85, p=0.01, Figure 2C). No sex effect was detected on 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).

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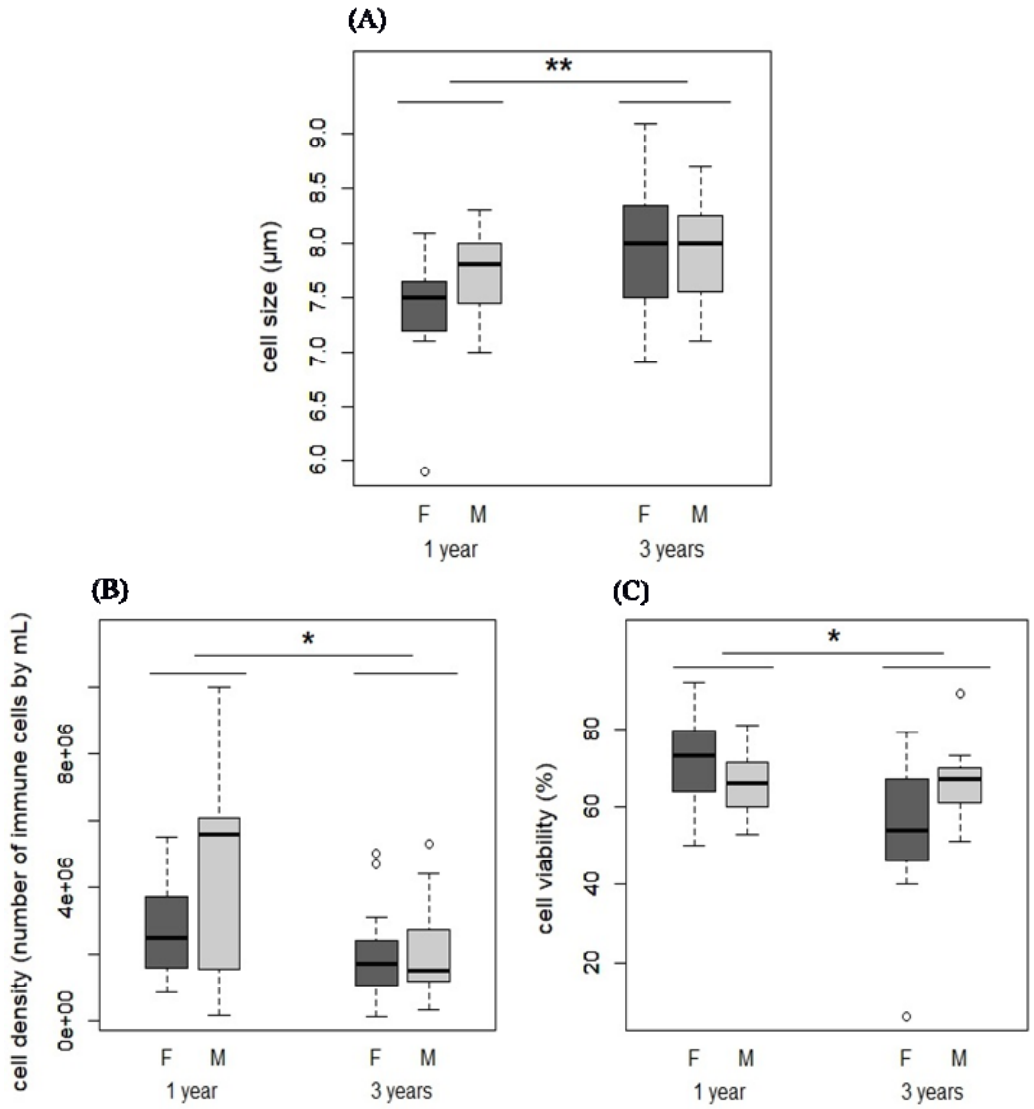

Figure 2: Immune cell size (A), density (B) and viability (C) according to age and sex in* A. vulgare *(F=females, M=males)

Thethick line depicts the median, the box the interquartile range, and the whisker are bounded to the most extreme data point within 1.5 the interquartile range. The outliers outside this range are displayed as open circles.N= 60 individuals: 15 1-year-old females, 15 1-year-old males, 15 3-years-old females and 15 3-years-old males. * denotes $p<0.05$ and ** denotes $p<0.01$

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TERT gene expression

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

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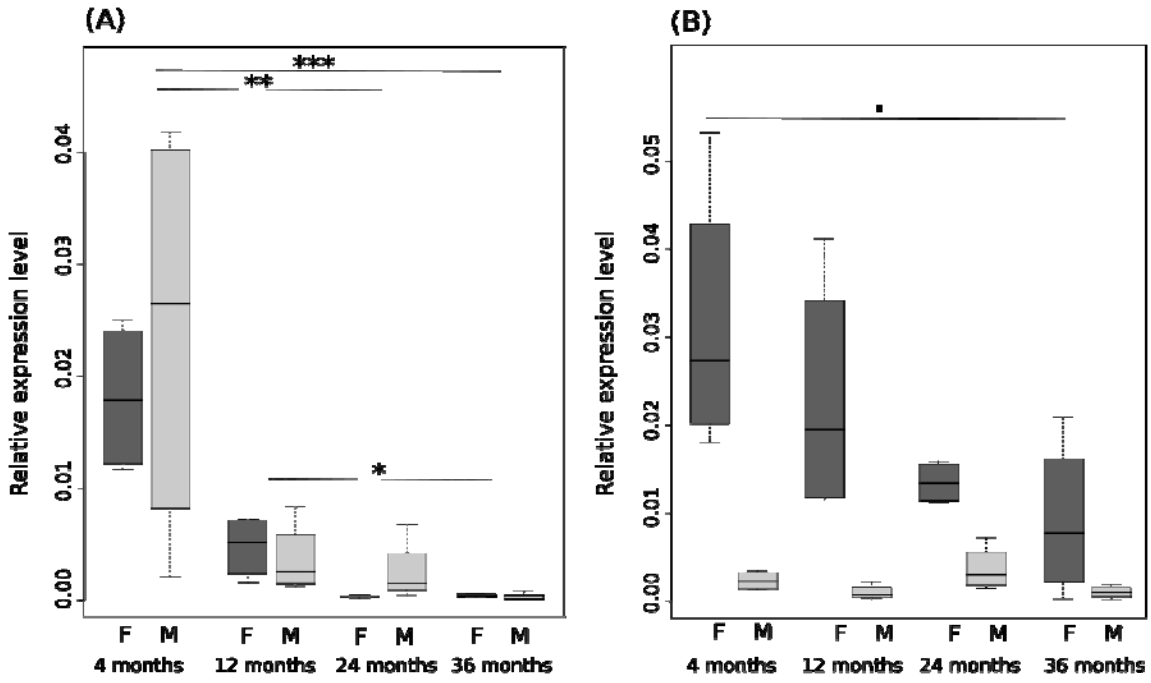

Figure 3: Relative expression level of TERT in (A) nerve cords and (B) in gonads in A. vulgare (F=females, M=males.

Expression of each gene was normalized based on the expression of Ribosomal Protein L8 (RbL8) and Elongation Factor 2 (EF2) as reference genes. The thick line depicts the median, the box the interquartile range, and the whisker are bounded to the most extreme data point within 1.5 the interquartile range. N= 176 individuals: 284-months-old 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 males, 20 3-years-old females, 20 3-years-old males. **.** denotes $p<0.10$, ** denotes $p<0.01$

4. Discussion

In this study, we tested several effective physiological biomarkers of vertebrate senescence to assess whether they could also be used in invertebrates such as the common woodlouse. Immune cells showed an increase in their size and a decrease in their density and

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

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

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

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

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

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

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

Acknowledgements

We would like to thank Sylvine Durand, Richard Cordaux, Isabelle Giraud and Bouziane Moumen for our constructive discussions as well as Marius Bredon, Carine Delaunay, Maryline Raimond and Alexandra Lafitte for technical assistance. The preprint of this work has been deposited in BioRxiv (https://doi.org/10.1101/583914).

Funding

This work was supported by the 2015–2020 State-Region Planning Contract and European Regional Development Fund and intramural funds from the Centre National de la Recherche

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Scientifique and the University of Poitiers. J.F.L. and J.M.G. are supported by a grant from the Agence Nationale de la Recherche (ANR-15-CE32-0002-01 to J.F.L.). This work has also received funding from the "Appel à projets de recherche collaborative inter-équipes 2016-2017" by the laboratory EBI.

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