Common biomarkers of vertebrate senescence respond to age variation in the invertebrate *Armadillidium vulgare*

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Declarations of interest: none

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 this context, we tested whether biomarkers of vertebrate ageing could be used to study senescence in a very promising invertebrate model of ageing: the common woodlouse *Armadillidium vulgare*. More specifically, we looked for the effect of age in woodlouse on three well established physiological biomarkers of ageing 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 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

Armadillidium vulgare, cellular senescence, invertebrates, Telomerase Reverse Transcriptase (TERT), β-galactosidase activity.

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).

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 (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 and di Fagagna 2007). As ageing is associated with cellular senescence (Herbig et al. 2006; Lawless et al. 2010; Wang et al. 2009), many biomolecular parameters potentially inform about senescence and can therefore be valuable tools for studying this process (de Jesus and 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 and Kindell 2005) to honeybees (Hsieh and 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 and Harley 1997; Shay and 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 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 (Lai et al. 2017; Podlevsky et al. 2007; Robertson and Gordon 2006).

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 (Ram and Costa 2018; Stanley 2012). 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 and 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 and Pitelka 1962). In addition, one study has already reported evidence of immuno senescence in this species (Sicard et al. 2010). Moreover, this indeterminate grower species moult every month, which suggests that patterns of cellular senescence differ between tissues.

In this context, we tested the suitability of β-galactosidase activity, immune cell parameters and the TERT gene expression to indicate 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 viability and density in *A. vulgare*. As males have higher adult survival than females (Paris and 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, females and males of each crosses are chosen to avoid consanguinity. Thus, the pedigree of each individual is available over several generations and studies on genetic diversity have shown good diversity in our breeding populations. For each clutch obtained, individuals were sexed, and brothers and sisters were separated to ensure virginity. Indeed, all individuals used in our experiment were maintained in the same laboratory conditions since our experiments and were all virgin. In our breeding, we observed that the maximal lifespan reach the 3 years, we are analyzing the survival in this species, in the meanwhile, we have arbitrarily established that before 12-months-old old, first year of reproduction, individuals are considered as “young

individuals", from 12-months old to 24 months old individuals are considered as "adult" and as we observed an increasing in mortality from 24 months-old to 36-months old so we qualify the 24-months old individuals as "old" individuals and the 36-months old as "very old" individuals. In common woodlouse approximately moult once a 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. In addition, we were limited to the available individuals in our breeding, we used 4 or 6-months old and 12-months old individuals as "young individuals" and as the "old animals" are sometimes not enough to be able to test several markers, in this case we chose to take either two years, or three years, knowing that two years is already an old age for the woodlice of our breeding.

2.2 Measure of β-galactosidase activity

Animals

To test the impact of age on the on β-galactosidase activity,180 individuals were used: 90 young (i.e. 6-months old12-months, 45 males and 45 females) and 90 old (24-months-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 and 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.

2.3 Measure 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. 12-months-old, 15 males and 15 females) and 30 old (36-months-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 concentrationcell concentration, 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 upstream (see Online Resources 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 (Online resource 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) 12-months-old, (3) 24-months-old and (4) 36-monthsold -old. Females and males were tested separately by pools of 5 individuals in 12--, 24-, 36-months-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 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 Online resource 1). Quantitative RT-PCR was performed using 0.6 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.

Concerning the immune parameters, linear models with Gaussian distribution were fitted to analyze variation in the cell size and viability. For the cell concentration, a linear model of the cell number (log-transformed, (Ives and 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 Tukey correction using R package PMCMR.

3. Results

β-galactosidase activity

The β-galactosidase activity was higher in old (i.e. 24-months-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).

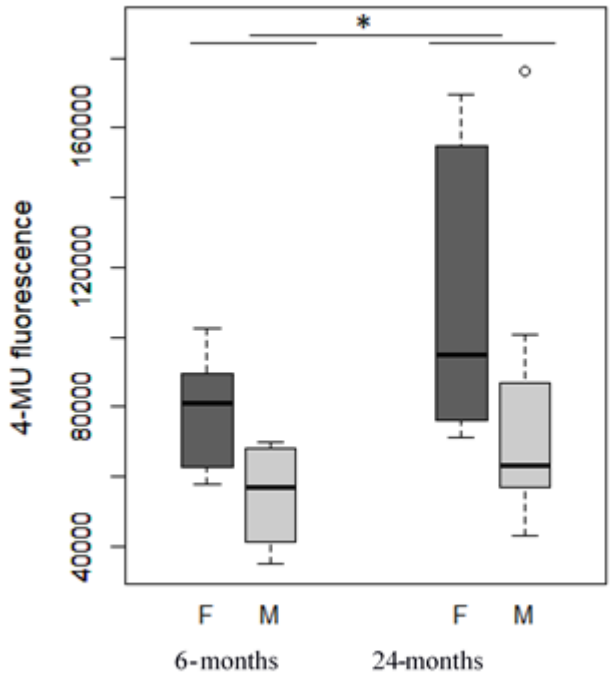

Fig. 1 β-galactosidase activity according to age and sex in A. vulgare (F=females, M=males)
The thick line depicts the median, the box the interquartile range, and the whisker are bounded to the most extreme data point within 1.5 the interquartile range. The outliers outside this range are displayed as open circles. N= 24 pools of 5 individuals. * denotes *p<0.05*

Immune cells parameters

Cell size was larger in 36-months-old than in 12-months2-months-old individuals ($F_{1,58}$=8.54, p=0.005, Figure 2A). Conversely, the cell concentration was higher in 12-months-old than in 36-months-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 36-months-old females ($F_{3,56}$=6.85, p=0.01, Figure 2C).

No sex ffect was detected on cell size ($F_{2,57}$=0.76, p=0.38, Figure 2A) or cell concentration ($F_{2,57}$=0.32, p =0.57, Figure 2B).

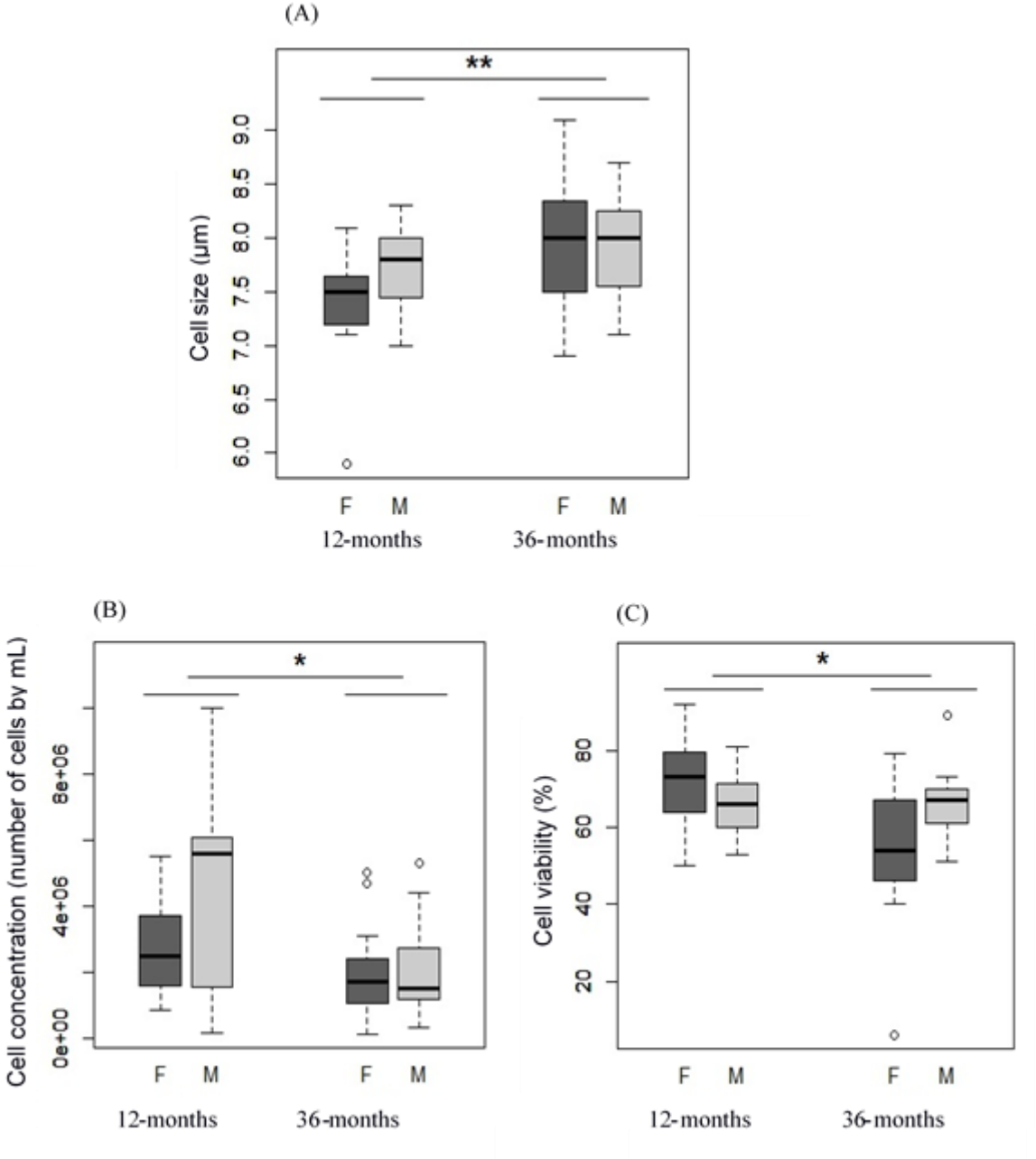

Fig. 2 Immune cell size (A), concentration (B) and viability (C) according to age and sex in A. vulgare (F=females, M=males)

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 12-months-old females, 15 12-months-old males, 15 36-months-old females and 15 36-months-old males. * denotes p<0.05 and ** denotes p<0.01

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 24-months-old and 36-months-old individuals (p=0.001 in both cases) and in 12-months-old individuals compared to 36-months-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 36-months-old females (p=0.054). In males, a general tendency was observed (χ^2_1=7.34, p=0.061, Figure 3B), the TERT gene expression tending to be higher in 24-months-old individuals compared to 12-months-old and 36-months-old individuals (p=0.14 and p=0.12, respectively, Figure 3B).

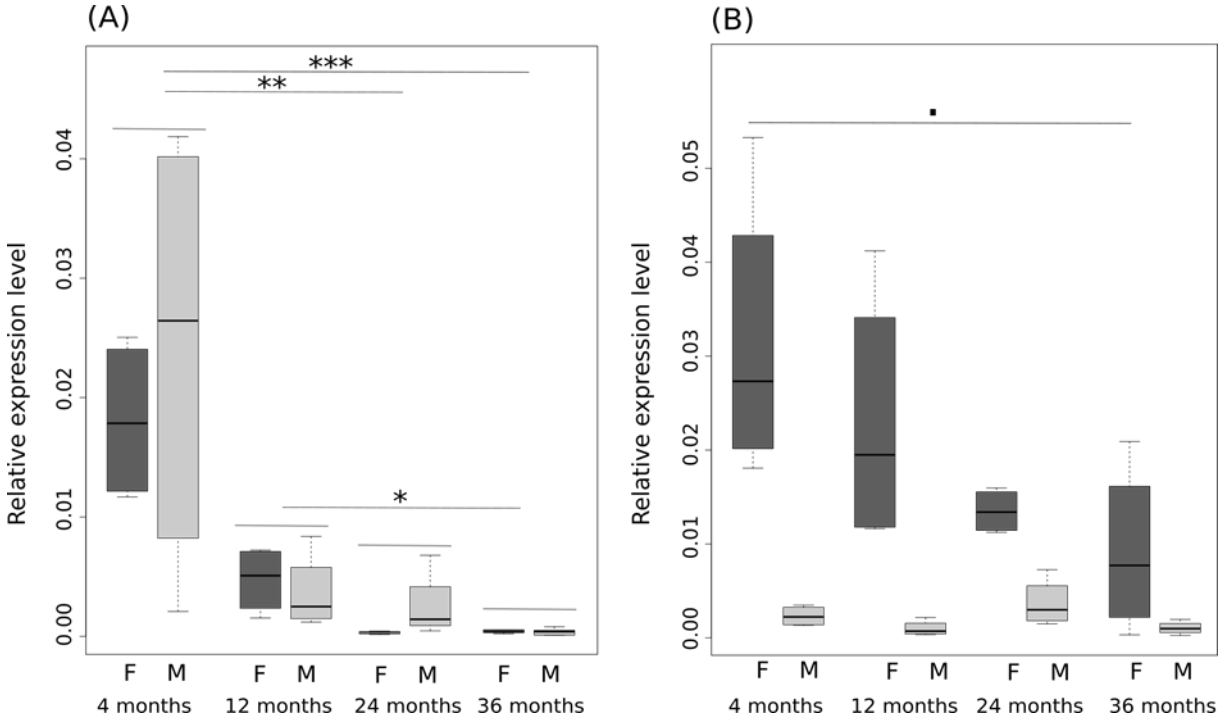

Fig. 2 Relative expression level of TERT in (A) nerve cords and (B) in gonads in A. vulgare (F=females, M=males). Expression of each gene was normalized based on the expression of Ribosomal Protein L8 (RbL8) and Elongation Factor 2 (EF2) as reference genes. The thick line depicts the median, the box the interquartile range, and the whisker are bounded to the most extreme data point within 1.5 the interquartile range. 176 individuals were used: 28 4-months-old females, 28 4-months-old males, 20 12-months-old females, 20 12-months-old males, 20 24-months-old females, 20 24-months-old males, 20 36-months-old females, 20 36-months-old males N=64 pools of 5 or 7 individuals. **.** denotes $p<0.10$, * denotes $p<0.05$, ** denotes $p<0.01$, *** denotes $p<0.001$.

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 as previously observed in crickets (*Gryllus assimilis*), a decrease in their concentration and viability with increasing age (Park et al. 2011). In nerve cords, the activity of the β-galactosidase enzyme increased as already observed in trophocytes and fat cells of honeybees (Hsieh and Hsu 2011).The TERT gene expression decreased with increasing age in nerve cords for the both sex and in gonads for females. In marine medaka *Oryzias melastigma*, the TERT expression, and the telomerase activity are low at the beginning (4-months) and at the end (12-months) of life, but high in mid- life (8 months) and a similar pattern tendency was also observed in male's woodlice gonads (Yip et al. 2017). In this study of marine medaka, the females have more TERT expression as in the woodlice female's gonads (Yip et al. 2017). These results shows both a strong link between age and TERT expression in the somatic lineage and indicates sex-dependent differences between tissues especially between somatic and germinal lineage.

Our study is in line with previous studies that have already revealed the possibility of using vertebrate biomarkers in invertebrates (Hsieh and 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 and 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. (Barja 2000; Barja and Herrero 2000; Finkel and Holbrook 2000; Harman 1956). In *A. vulgare*, the 24- and 36-months-old individuals could have therefore accumulated more cellular damages during their lifetime, leading to the cellular senescence we report.

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 and 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 (Marais et al. 2018; Tidière et al. 2015). One of the main theory proposed to explain sex differences in longevity or senescence patterns relies on different resource allocation strategies between sexes (Bonduriansky et al. 2008; Vinogradov 1998).The shorter lifespan in females *A. vulgare*, that allocate more energy to reproduction than males (Paris and 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 and 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 could correspond to biomarkers of senescence in woodlouse (Baker and 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 ageing. 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, available genome, renewal of certain tissues and sex-dependent strategies in terms of reproduction, could be a model of choice to study sex-specific actuarial and cellular senescence.

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 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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