Development of senescence biomarkers in the common woodlouse

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Abstract

Over time, damages accumulate in the cells leading to the process of cell senescence. Many cellular modifications can then attest to this process and are called senescence biomarkers. Senescence biomarkers are highly studied in humans and are particularly useful for understanding the processes involved in age-related diseases. However, while studies on ageing are increasingly focusing on invertebrate models, senescence biomarkers remain poorly

developed in these organisms. In this study, we looked at the effect of age on three known biomarkers in vertebrates: immune cells (cell size, density and viability), beta-galactosidase activity and telomerase expression on *A. vulgare*, a terrestrial isopod. As expected, the size of immune cells was higher in older individuals while their density and viability decreased, β-galactosidase activity increased with age while Telomerase Reverse Transcriptase (TERT) gene expression decreased. These biomarkers classically used in vertebrates are thus correlated with age in our invertebrate model and make good biomarkers to highlight cell senescence in *A. vulgare*. A strong gender effect was also observed on markers and could indicate different resource allocation strategies. This last point encourages us to use the biomarkers developed here to understand the other factors involved in the diversity of senescence patterns observed in living world.

Keywords

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

1.Introduction

The cellular senescence corresponds to the progressive deterioration of the cell leading the stop of the cellular cycle (Campisi and di Fagagna, 2007). The principal hypothesis proposed to explain this deterioration is the oxidative stress hypothesis also known as free radical theory of ageing (Finkel and Holbrook, 2000; Wickens, 2001). Among this theory, oxygenated reagents lead an accumulation of damage modifying the integrity and functions of lipids, proteins as well as DNA and mitochondria in the cell (Finkel and Holbrook, 2000; Terman and Brunk, 2006). Many studies have examined senescence and highlighted parameters related to this phenomenon (De Jesus and Blasco, 2012).

The observation of cells is the first clue of cellular senescence. The senescence processes lead to an increase in the size of the senescence cell which becomes larger than a non-senescent cell (Hayflick, 1965; Rodier and Campisi, 2011). The evolution of the efficacy and integrity of cells implied in immunity has been studied many times and has shown effects of age, making them particularly relevant to study cellular senescence: for example, a diminution of the number of effective immune cells with age has been reported in wild vertebrates (Cheynel et al., 2017) but also in invertebrates including mosquitoes *Aedes aegypti* (Hillyer et al., 2004) and crickets *Gryllus assimilis* (Park et al., 2011).

The activity of proteins could also be related to cellular senescence. Among them, the most popular is the activity of the lysosomal β-galactosidase enzyme, which increases when the cell enters in senescence (Dimri et al., 1995; Itahana et al., 2007). This phenomenon was observed in senescent cells of many organisms ranging from humans (Gary and Kindell, 2005) to honeybees (Hsieh and Hsu, 2011). Another target protein is the telomerase, enzyme that lengthens the ends of telomeres, essential structures at the end of chromosomes to protect the integrity of the genome, which shorten with each cell division and conduct cell senescence as when their telomeric size becomes critical (Chiu and Harley, 1997; Shay and Wright, 2005). The activity of telomerase depends of organism, age but also tissues. In humans, telomerase is active during the development before the birth and after lonely in stem and germ cells (Liu et al., 2007; Morgan, 2013) while in the long-lived species *Daphnia pulicaria*, the telomerase activity in all tissues of the body decreases with age (Schumpert et al., 2015). The Telomerase Reverse Transcriptase (TERT) gene was detected in numerous organisms including vertebrates, fungi, ciliates and insects (Robertson and Gordon, 2006). As it has been shown to be related to cell survival in humans (Cao et al., 2002), it could thereby be directly link to cellular senescence in others organisms.

β-galactosidase activity, immune cell parameters (i.e. size, density and viability) and telomerase activity do not directly reflect the age of the organisms, but the senescence process itself; thus, they are classically considered as senescence biomarkers (Baker and Sprott, 1988). These biomarkers are very useful for understanding the processes involved in senescence and are widely used to understand processes of age-related diseases in humans (Hoos et al., 1998; Xia et al., 2017). Although their usefulness is not in doubt, they are never used to understand ageing patterns variability observed across and within species and still poorly studied in invertebrates. However, the use of both these senescence biomarkers and invertebrate models could meet the challenges of studies on factors impacting ageing variability that require long-term monitoring using lots of replicates under controllable environmental conditions. Besides, invertebrates are increasingly coveted in experimental approaches in the senescence study because they have important similarities with vertebrates in terms of senescence process, but also because they are more easy to manipulate experimentally and can be monitored throughout their entire lifetime (Ram and Costa, 2018).

In this context, we developed in a long-lived invertebrate, the common woodlouse *Armadillidium vulgare*, the senescence biomarkers presented above (β-galactosidase activity, immune cell parameters and telomerase activity) to be used in future studies on factors involved in ageing variability. According to the literature, we expected in *A. vulgare* both an increase in β-galactosidase activity, a decrease of immune cell viability and density and a decrease of TERT expression with increasing age. Sex specific patterns were also expected on these senescence biomarkers.

2. Materials & Methods

2.1. Biological model

Individual *A. vulgare* used in the following experiments were derived from a wild population collected in Denmark in 1982. Individuals have been maintained on moistened soil with the natural photoperiod of Poitiers (France 46.58°N, 0.34°E, 20°C) at 20°C with food (i.e. dried linden leaves and carrots) *ad libitum.* 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 moult throughout their lives according to a molting cycle (Lawlor, 1976). At 20°C, they approximately moult once per month (Steel, 1980) and all the cells of the concerned tissues are renewed. However, the brain, the nerve cord and gonads are not part of tissues renewed during molting and are therefore good candidates for tissue-specific study of senescence in this species. Males and females were tested separately to test the sex impact on candidates' biomarkers.

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-old, 45 males and 45 females) and 90 old (2-years-old, 45 males and 45 females).

Protocol

Individuals were dissected separately in Ringer (Sodium Chloride 394 mM, Potassium Chloride 2 mM, Calcium Chloride 2 mM, Sodium Bicarbonate 2 mM) and nerve cord was removed. Nerve cords were chosen because they are not regenerated during molting. To obtain a sufficient quantity 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). Samples 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 were homogenized at 0.1 mg/mL. The β-galactosidase activity was measured as previously described by Gary and Kindell (2005). Briefly, 100 µL of protein extract 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 measurement. 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.

Statistics

The β-galactosidase activity was analyzed with linear mixed effect models using the R software (R. Core Team, 2016) and the R 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 and age and sex and their two-way interactions as fixed factors.

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) that were candidates for providing biomarkers of senescence 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).

Protocol

To study the impact of age on the immune parameters, 3 µL of haemolymph were collected per individual. A hole was bored in the middle of the 6th segment and 3 µL of haemolymph were collected with an eyedropper and deposited promptly in 15 µL of 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 permit the coloration of dead cells. Thereafter, 10 µL of this solution were deposed in (Invitrogen Coutness®) counting slide (Thermofisher). The immune cell density, the immune cell viability and the immune cell size were evaluated using an automated Cell Counter (Invitrogen Countess®).

Statistics

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 and Freckleton Robert, 2015) was fitted using the R software (R. Core Team, 2016).

2.4 Measure of TERT expression

The identification of the Telomerase Reverse Transcriptase (TERT) was firstly performed from the annotation of the ongoing *A. vulgare* genome project (Chebbi et al., 2019). In order to verify whether this gene was present and preserved in this gene in crustaceans, phylogenetic analyses were carried out upstream, all of these analyses are presented in Supplementary materials 1, 2 and 3. This gene has been found in most crustacean transcriptomes and was very well conserved within the phylogenetic tree (Supplementary material 3), thus a significant role of the TERT is expected.

2.4.1 Genes expression

Animals

To test the effect of age in the expression of TERT gene, we needed 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 of males and 4 replicates of females. 176 individuals were used for this experiment. For each group we tested the expression level of the TERT in two different tissues: the nerve cord and gonads.

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 an homogenisation using a Vibra Cell 75,185 sonicator (amplitude of 30%). RNA was quantified by NanoDrop technology and at -80°C until their use. Reverse transcriptions (RT) were made from 500ng of RNA previously extracted and using the kit SuperScript^{TM} IV Reverse Transcriptase (Thermo Fisher Scientific) according to the supplier's instruction. Primers were designed using the identified gene: primer TERT_F: 5'-AGGGAAAACGATGCACAACC-3' and primer TERT_R: 5'-GTTCGCCAAATGTTCGCAAC- 3'. Quantitative RT-PCR was performed using 0.6 µl of each forward and reverse 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 in 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

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 the R software (R. Core Team, 2016) and the R package PMCMR.

3. Results

β-galactosidase activity

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

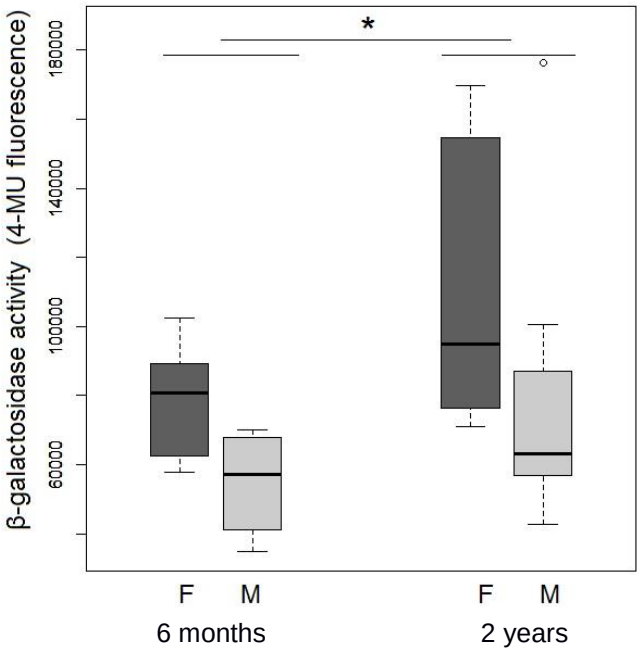

Figure 1: β-galactosidase activity according to age and sex in *A. vulgare* (F=females, M=males)

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. * *p<0.05*

Immune cells parameters

Age had a statistically significant effect on cell size ($F_{1,58}$=8.54, p=0.005, Figure 2A). Cell size was larger in 3-years-old than in 1-year-old individuals. 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).

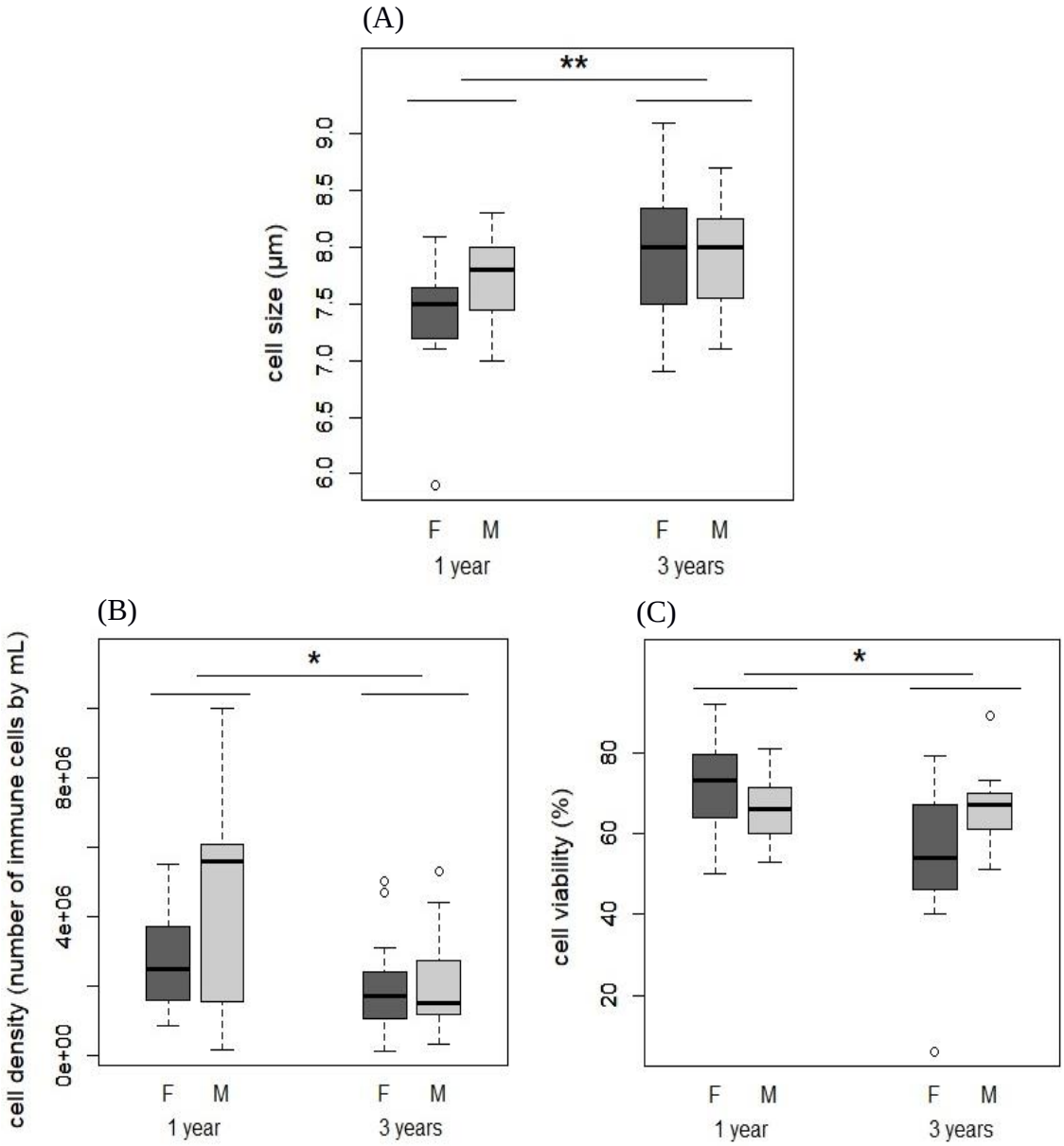

Figure 2: Immune cell size (A), density (B) and viability (C) according to age and sex in *A. vulgare* (F=females, M=males)

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= 60 individuals: 15 1-year-old females, 15 1-year-old males, 15 3-years-old females and 15 3-years-old males. * $p<0.05$, ** $p<0.01$

TERT expression

The TERT expression decreased with age in nerve cords (X^2_3=23.30, p<0.001, Figure 3A). More precisely, the TERT expression was higher in 4-months-old individuals comparing to 2-years-old individuals and 3-years-old individuals (Respectively: p=0.001, p<0.001) and in 1-year-old individuals comparing to 3-years-old individuals (p=0.038), without sex impact (X^2_1=0.14, p=0.70, Figure 3A). In gonads, the TERT expression was strongly higher in females (X^2_1=17.81, p<0.001, Figure 3B) and tended to decrease with age (X^2_3=7.5, p=0.057, Figure 3B) as the TERT expression tended to be higher in 4-months-old females comparing to 3-years-old females (p=0.054). In males, a general tendency was also observed (X^2_1=7.34, p=0.061, Figure 3B), the TERT expression tending to be higher in 2-years-old individuals comparing to 1-year-old individuals and 3-years-old individuals (Respectively: p=0.14, =0.12).

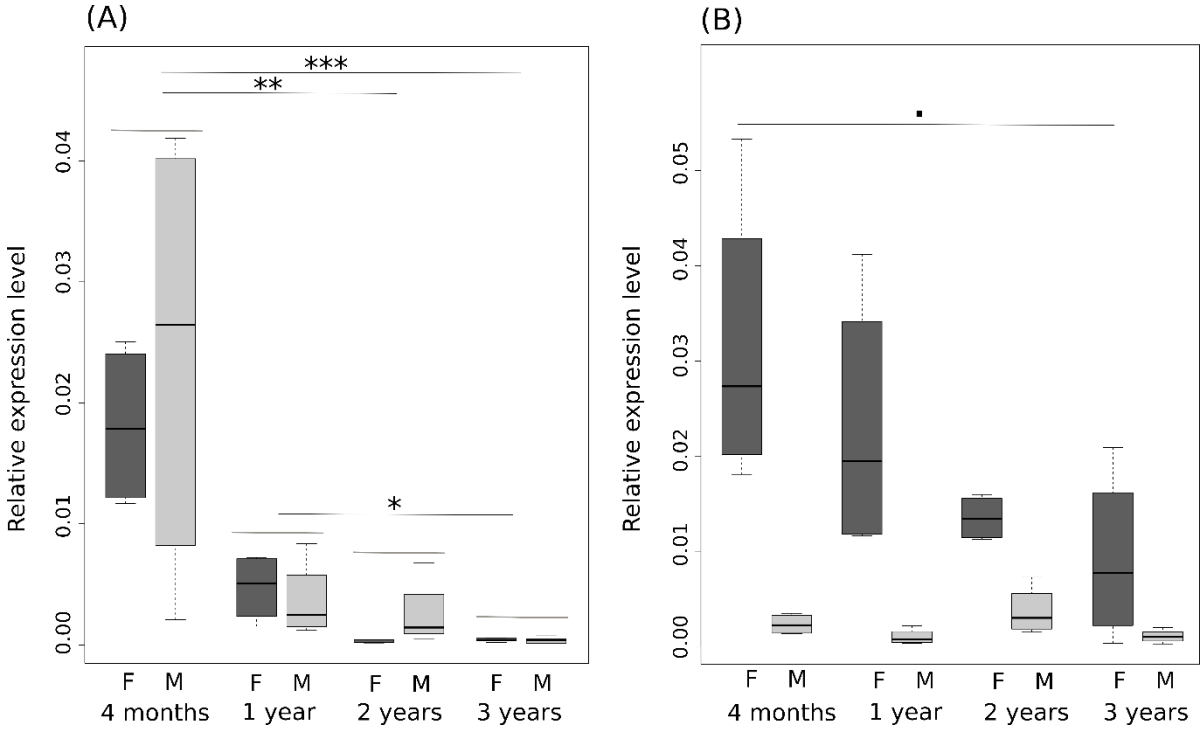

Figure 3: Relative expression level of TERT in (A) nerve cords and (B) in gonads in *A. vulgare* (F=females, M=males).

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: 28 4-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.

. $p<0.10$, ** $p<0.01$

4. Discussion

As expected, immune cells showed an increase in their size and a decrease in their density and viability with age. In nerve cords, the activity of the β-galactosidase enzyme increased while the TERT gene expression decreased with age. These results prove the presence of increasing cellular senescence in *A. vulgare* with age. In the gonads, TERT expression was too low in males and did not decrease sufficiently in both sexes to provide good biomarkers of senescence. Our study is not the first evidence that vertebrate biomarkers can be useful in

invertebrates as the β-galactosidase enzyme also increases in honeybees *Apis mellifera* with age (Hsieh and Hsu, 2011), the immune cells also decreases in density in *Aedes aegypti* (Hillyer et al., 2004) and crickets *Gryllus assimilis* (Park et al., 2011) and the telomerase activity decreases with age in *Daphnia pulicaria* (Schumpert et al., 2015). By testing a set of different biomarkers, often studied independently, our study supports the idea that routine biomarkers used in vertebrates can be adapted in invertebrates and confirms that the senescence process is quasi-ubiquitous in living world and can be expressed in a similar way in very different organisms.

Previously study showed that the probabilities to survive decreases with age in *A. vulgare* (Paris and Pitelka, 1962). The damage 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 2- and 3-years-old individuals could have therefore accumulated more damages during their life leading the cellular senescence observed.

Our present study also showed a strong difference in cellular senescence patterns on biomarkers between males and females, females presenting 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 remain to be quantified in *A. vulgare* but recent reviews have revealed that such differences are common in both vertebrates and invertebrates (Tidière et al. 2015; Marais et al. 2018 for reviews). One of the main theory proposed to explain sex differences in longevity senescence patterns relies on different resource allocation strategies between sexes (Bonduriansky et al., 2008; Vinogradov, 1998), which was already evoked to explain the shorter lifespan observed in females *A. vulgare* (Paris and Pitelka, 1962).

The resources allocation strategies proposed to explain sex differences could also be disturbed by environment (Shertzer and Ellner, 2002). As our biomarkers were able to highlight differences between sexes, they will probably constitute useful tools to underline other factors (e.g. environment stressors) involved in the diversity of senescence patterns observed in *A. vulgare* and more generally in the living world.

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