Development of senescence biomarkers in the common woodlouse

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Abstract

During their lives, organisms accumulate, damages in their cells leading to a process called cellular senescence. Senescent cells display many cellular modifications that can be assessed through different biomarkers. Senescence biomarkers are highly studied in humans and are particularly useful to understand the processes involved in age-related diseases. However, while studies on invertebrate ageing are increasing, senescence biomarkers remain

poorly developed in these taxa. In order to develop useful senescence biomarkers in invertebrates and more particularly in the common woodlouse *Armadillidium vulgare*, we looked at the effect of age on three known biomarkers 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 on this species. As expected, we observed that the size of immune cells was higher in older individuals while their density and viability decreased, the β-galactosidase activity increased with age while the Telomerase Reverse Transcriptase (TERT) gene expression decreased. These biomarkers classically used in vertebrates are thus correlated with age in our invertebrate model and make reliable biomarkers to highlight cell senescence in *A. vulgare*. A strong gender effect was also observed on senescence biomarkers that could indicate different resource allocation strategies. These biomarkers, allowing us to highlight differential cellular senescence between males and females, could be a new tool to test the impact of different factors influencing life history traits and related resources allocation and consequently help us to understand the diversity of senescence patterns observed between individuals, sexes, populations and species.

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 main hypothesis proposed to explain this deterioration relies on the oxidative stress hypothesis, also known as free radical theory of ageing (Finkel and Holbrook, 2000; Wickens, 2001). According to this theory,

oxygenated reagents lead to an accumulation of damages, modifying the integrity and functions of lipids, proteins as well as DNA 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).

One of the prominent characteristic of senescent cells is an increase in the cell size which becomes larger than a non-senescent cell (Hayflick, 1965; Rodier and Campisi, 2011). The evolution of the integrity and efficiency of immune cells is particularly relevant to study cellular senescence as a diminution of the number of effective immune cells with age has been reported in vertebrates (Cheynel et al., 2017) and invertebrates including mosquitoes *Aedes aegypti* (Hillyer et al., 2004) and crickets *Gryllus assimilis* (Park et al., 2011). Another marker used to study cellular senescence is the enzymatic activity of the β-galactosidase. This enzyme is an hydrolase which 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 was observed in senescent cells of many organisms ranging from humans (Gary and Kindell, 2005) to honeybees (Hsieh and Hsu, 2011). Another protein involved in the cellular senescence process is the telomerase, a ribonucleoprotein complex composed by two essential components, the telomerase reverse transcriptase (TERT) and the telomerase RNA (TR) and others accessorial proteins (Podlevsky et al., 2007). Telomerase lengthens the ends of telomeres, the latter are structures located at the end of chromosomes which protect chromosome integrity and shorten after each cell division. Cell senescence arises when the telomere size becomes critically short (Chiu and Harley, 1997; Shay and Wright, 2005).The telomerase activity depends on organism, age and also tissues. In humans, telomerase is active during the development before the birth and after only in stem and germ cells (Liu et al., 2007; Morgan, 2013) while in the *Daphnia pulicaria*, the telomerase activity in all tissues of the body decreases with age (Schumpert et al., 2015). As the TERT is essential

in the telomerase protein complex it has been shown to be related to cell survival in humans (Cao et al., 2002) and was detected in numerous species including vertebrates, fungi, ciliates and insects (Podlevsky et al., 2007; Robertson and Gordon, 2006)

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 often used to understand the processes involved in senescence and age-related diseases in humans (Hoos et al., 1998; Xia et al., 2017). Although their usefulness is not in doubt, they are never been used to understand variability of ageing patterns observed across and within species. Invertebrates are increasingly coveted in experimental approaches in senescence studies. Indeed, they share similarities with vertebrates in terms of senescence processes but also because it is easier to manipulate them experimentally and they can be monitored throughout their entire lifetime (Ram and Costa, 2018). These advantages make them models of choice for the development of senescence biomarkers.

In this context, we developed in a long-lived invertebrate (3-4 years old), the common woodlouse *Armadillidium vulgare*, the senescence biomarkers described above (β-galactosidase activity, immune cell parameters and the TERT gene expression) to be used in future studies on factors involved in ageing variability. According to the literature and over the time, we expected an increase in β-galactosidase activity, a decrease of both TERT gene expression and immune cell viability and density in *A. vulgare*. As at the adulthood, males have a greater 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, able to live up to three years (Paris and Pitelka, 1962) 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 (with 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 molt throughout their lives, with approximately on moult per month At 20°C (Steel, 1980), during this process all the cells of the concerned tissues are renewed. 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 on our candidate 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) individuals.

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. 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 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 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) 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) 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 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 color the 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®).

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 verify whether this gene was present and preserved in crustaceans, phylogenetic analyses were carried out 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 significant and conserved role of the TERT gene.

2.4.1 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 quantified by NanoDrop technology

and was stored at -80°C until used. 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 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 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 interactions as fixed factors.

Concerning the immune parameters, linear models with Gaussian distribution were fitted to analyze variations 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.

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 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 significantly higher β-galactosidase activity in females than in males (X^2_1=7.26, p=0.007, Figure 1).

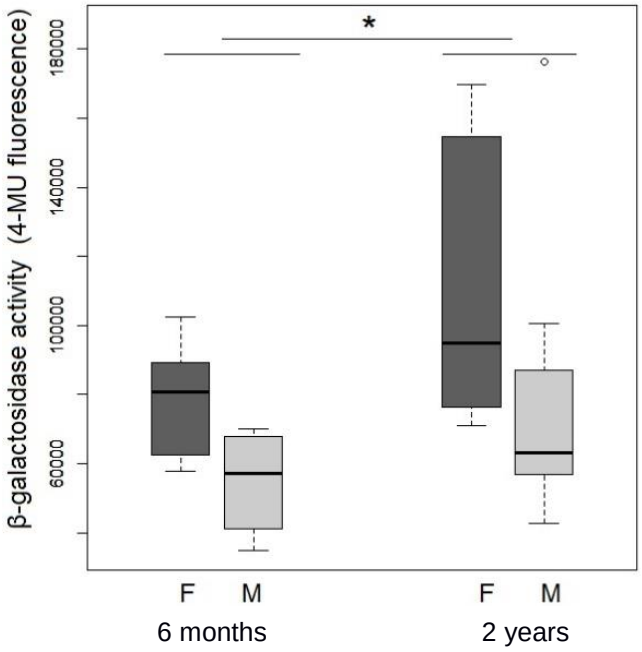

Figure 1: β-galactosidase activity according to age and sex in *A. vulgare* (F=females, M=males)

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. * denotes *p<0.05*

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).

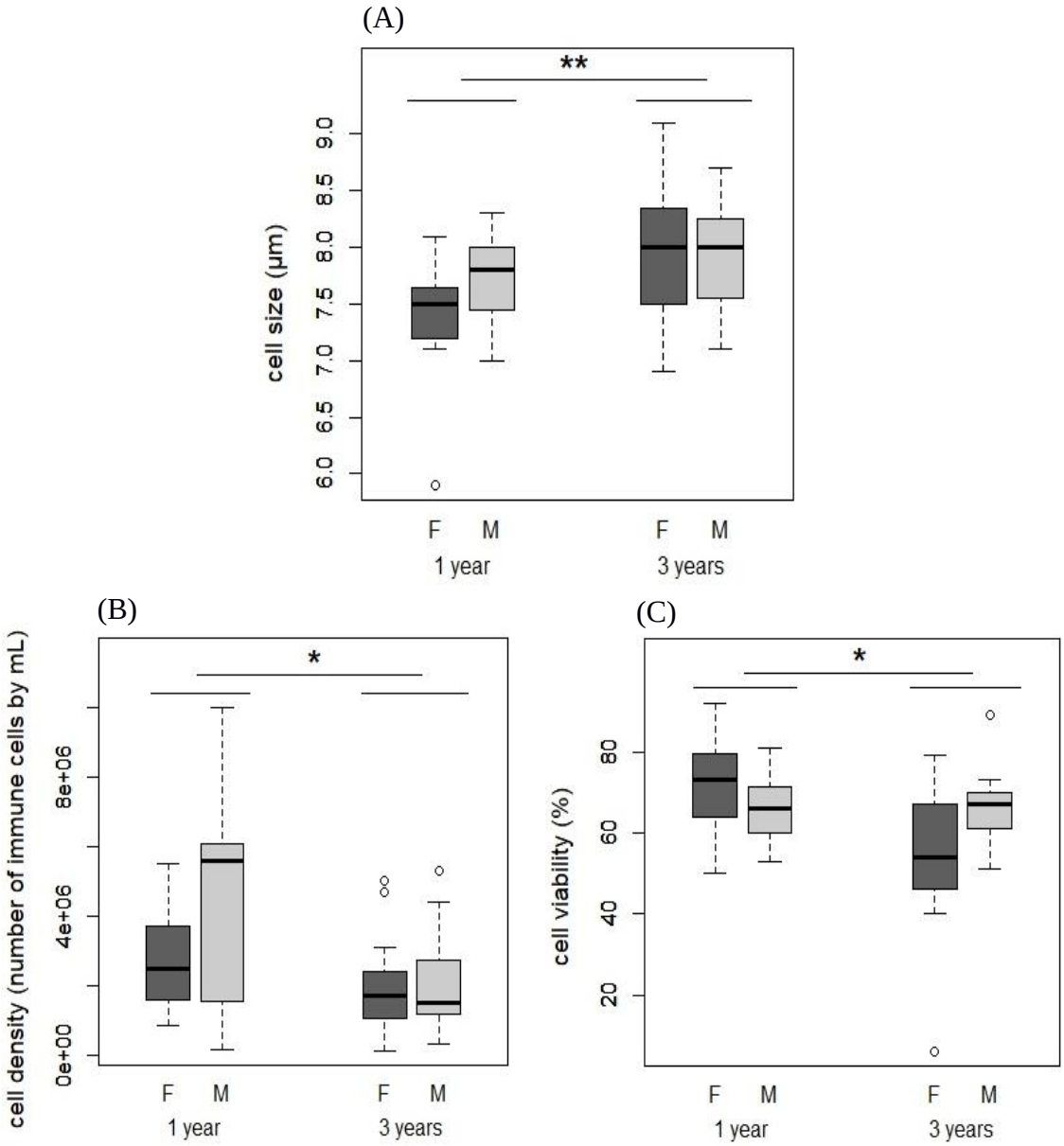

Figure 2: Immune cell size (A), density (B) and viability (C) according to age and sex in *A. vulgare* (F=females, M=males)

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. * denotes p<0.05 and ** denotes p<0.01

TERT gene expression

The TERT gene expression decreased with age in nerve cords (X^2_3=23.30, p<0.001, Figure 3A). More precisely, the TERT gene 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 effect (X^2_1=0.14, p=0.70, Figure 3A). In gonads, the TERT gene 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 gene 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 gene expression tending to be higher in 2-years-old individuals but not significantly comparing to 1-year-old individuals and 3-years-old individuals (Respectively: p=0.14, p=0.12).

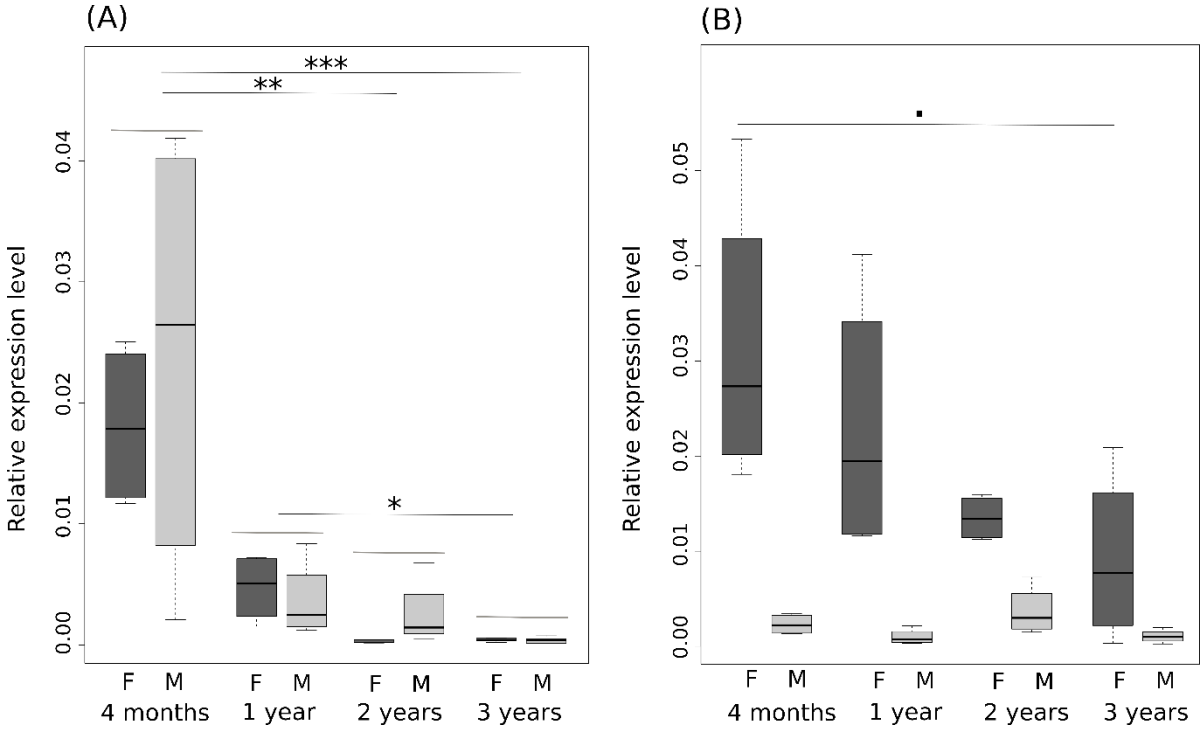

Figure 3: Relative expression level of TERT in (A) nerve cords and (B) in gonads in *A. vulgare* (F=females, M=males).

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.

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

4. Discussion

In this study, we tested several effective senescence biomarkers on vertebrates to see if they could also be used in our invertebrate model: the woodlouse. 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 support the presence of increasing cellular senescence in *A.*

vulgare with age. In the gonads, the TERT gene expression was too low in males and was not sufficiently variable between sexes to provide good senescence biomarker in this tissue.

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 biomarkers, often studied independently, our study confirms the idea that routine biomarkers used in vertebrates can be adapted in invertebrates and supports 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 decrease with 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 2- and 3-years-old individuals could have therefore accumulated more cellular damages during their life leading the cellular senescence observed.

Our present study also showed a strong difference between sexes concerning the cellular senescence patterns on biomarkers: females present 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 such differences are quite common both in 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* as they invest more energy in reproduction than males (Paris and Pitelka, 1962).

The resources allocation strategies proposed to explain sex differences could also be driven by environment (Shertzer and Ellner, 2002). As our senescence biomarkers were able to highlight differences between sexes, and as support in Depeux et al., 2019, they could constitute useful tools to underline other factors (e.g. environment stressors) involved in the diversity of senescence patterns observed between individuals, sexes, populations and species.

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