



Effect of lifespan and age on reproductive performance of the tardigrade *Acutuncus antarcticus*: minimal reproductive senescence

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Abstract Reproductive output is one of the central attributes of life history, and knowledge of age-specific reproduction can enhance the understanding of population performance and dynamics. Tardigrades are microscopic invertebrates that live in marine, freshwater and terrestrial ecosystems. While changes in fertility in relation to age are known to occur in other invertebrate groups, the subject has not been specifically addressed in tardigrades. The current study demonstrates for the first time the effect of lifespan and age on reproductive characteristics of the tardigrade species, *Acutuncus antarcticus* (Richters 1904), based on the observation of individuals over their entire lifespan under constant environment conditions in the laboratory. Clutch size of *A. antarcticus* fluctuated conspicuously throughout individual lifespans. Weak effects of age were observed on

oviposition interval and hatching success, with the former increasing slightly and the latter decreasing slightly with age. Numbers of oviposition events and eggs produced per individual varied greatly and were correlated with lifespan. No significant relationships between clutch size, hatching time or hatching success with lifespan were detected. The majority of the individuals continued oviposition until shortly before death, with no suggestion of a post-reproductive lifespan. Our observations suggest that any decline in fertility with age in this species of tardigrade is minimal. The study provides new insight into the reproductive biology and ageing of invertebrate species.

Keywords Ageing · Fertility · Tardigrada · Antarctica

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Introduction

Reproductive output is one of the central attributes of life history, providing measures of individual and population performance (Lester et al., 2007). It consequently influences patterns of population persistence and contributes to the understanding of species range boundaries (Brown & Lomolino, 1998; Gaston, 2003). Describing reproductive strategies and assessing fecundity are considered fundamental topics in the study of biology and population dynamics (Hunter

et al., 1992). Moreover, knowledge of age-specific reproduction can enhance the understanding of population performance and dynamics associated with intrinsic and extrinsic factors, as well as regulatory mechanisms (Del Giudice et al., 2007). Patterns of senescence are highly variable among species and change in fertility in relation to age is also known to vary considerably across different groups of organisms (Jones et al., 2014).

Tardigrades are microscopic invertebrates that live in marine, freshwater and terrestrial ecosystems. In terrestrial habitats, animals are active only when they are surrounded by a film of water (Bertolani et al., 2009). Their ability to enter a cryptobiotic state, in which they can resist physical stresses by effectively switching off metabolism (Keilin, 1959), allows tardigrades to live like aquatic organisms in terrestrial environments and to survive and evolve under sometimes drastic seasonal and diurnal changes in their habitats (Bertolani, 1994; Rebecchi et al., 2007). Marine tardigrades are generally gonochoristic, while the most common mode of reproduction in limno-terrestrial species is parthenogenesis although gonochorism and hermaphroditism occur in some species (Bertolani, 2001). Parthenogenesis in tardigrades generally occurs through apomixis (ameiotic maturation of the oocytes) and is also linked to polyploidy (Bertolani, 1994).

Some data and interpretation relating to reproduction, development and generation time in tardigrade species in natural populations are available (Morgan, 1977; Kathman & Nelson, 1987; Schuetz, 1987; Schuster & Greven, 2007, 2013). In addition to the few earlier laboratory studies focused on life history (Baumann, 1964; Dougherty, 1964), traits of several tardigrade species in culture have been described in detail in the last decade (Altiero & Rebecchi, 2001; Suzuki, 2003; Altiero et al., 2006, 2010, 2015; Hohberg, 2006; Lemloh et al., 2011; Schill, 2013; Tsujimoto et al., 2015). These studies quantified species-specific or population-specific traits including lifespan, age at first oviposition, clutch size, total number of oviposition events, the number of eggs produced per individual and egg development time. However, they did not generally address the effect of lifespan or age on reproductive characteristics.

Without knowledge of changes in fertility or other reproductive parameters over the lifespan, understanding of species-specific or population-specific

characteristics of reproduction, thus of the reproductive performance and population dynamics of tardigrade species, remains limited. Therefore, in this study, survival and reproduction of individuals of the tardigrade, *Acutuncus antarcticus* (Richters, 1904), were studied in detail over their entire lifespan under constant laboratory conditions. The data obtained permit discussion of reproductive senescence of *A. antarcticus* in association with ageing, expanding knowledge of reproductive biology in invertebrate groups.

Materials and methods

Study species

This study used a culture of the eutardigrade *A. antarcticus* (Richters, 1904) (Hypsibiidae), originally established in the laboratory in 2013 from a phyto-benthos sample collected from Hamagiku–Ike Lake in Skarvsnes (69°28'S 39°39'E) on the Sôya Coast of East Antarctica (Tsujimoto et al., 2015). *Acutuncus antarcticus* is known to be herbivorous (Dougherty, 1964; Kagoshima et al., 2013). The 68 individuals and their reproductive traits considered in this study are those preliminarily analysed by Tsujimoto et al. (2015). Automictic (thelytokous meiotic) parthenogenesis has been reported recently from a different population of the same species (Altiero et al., 2015).

Culture conditions

Sixty-eight juveniles hatched from eggs randomly selected from the established culture within a 24-h period at 15°C were reared in individual wells on culture plates at 15°C in the dark. The temperature of 15°C was selected as being that which generated the maximum reproductive output of an *A. antarcticus* sub-sample over a 17-day period (Tsujimoto et al., 2015). Practical limitations on the number of individuals available, and the need to maximize replication meant that we focused our observations at this temperature and were able to obtain a sample size larger than 50 individuals. TPP[®] tissue culture plates (12 wells, flat bottom) were used with a layer of 300 µl of 1.5% agar gel on the bottom of each well. As a food source, 600 µl of Volvic[®] water and 1.8 µl of a suspension of *Chlorella* sp. (purchased every three

weeks from Chlorella Industry Co.) were added to each well. Animals were transferred to new culture dishes and provided with the food source every week, as it is known that overgrowth of bacteria and/or fungi that are problematic in rearing tardigrades can be avoided in this way (Altiero & Rebecchi, 2001). Additionally, cultures were stored in the dark so as to avoid overgrowth of the algal food source. There was no suggestion of any problems arising from rearing these animals under constant dark conditions.

Lifespan and reproductive traits

Individual tardigrades were inspected daily and their survival and egg production were monitored. Eggs from each clutch were isolated on the day of oviposition, separated and transferred individually to wells on new culture plates. Subsequent hatching of the isolated eggs was monitored daily until 30 days after oviposition. Data on lifespan, timing and clutch size of each oviposition event, egg development time to hatching (hatching time) and hatching success were recorded. Observations were made using a dissecting microscope (Olympus SZX7) at $\times 56$ magnification.

Statistical analysis

As not all the data obtained were normally distributed, Kendall's tau statistic was used to determine the correlations of reproductive traits (total number of oviposition events, total number of eggs, age at first oviposition, days from last oviposition to death, clutch size, oviposition interval, hatching time and hatching success) of individuals with their lifespans. Correlations of oviposition intervals, and egg hatching times and success with clutch size were also calculated. Generalized additive mixed models (GAMMs) with an identity link function and a thin plate regression spline smoothing function for estimation (Lin & Zhang, 1999) were applied to analyse the effect of age on clutch size, oviposition interval, hatching time and hatching success. For GAMM analyses, random intercept models were applied to account for variation between individual tardigrades. R statistical software, version 3.1.1, was used in all the statistical analyses with 'gamm4' package for GAMM.

Results

Lifespan and lifetime reproduction of *Acutuncus antarcticus*

Under the laboratory rearing environment at 15°C, the mean and the median lifespans of *A. antarcticus* individuals were 69.2 ± 36.4 and 65 days, respectively (Fig. 1; Table 1). Lifespan, total number of oviposition events and total number of eggs deposited by individuals varied greatly (though all had right-skewed distributions), while age at first oviposition was predominantly 9 or 10 days (Fig. 2; Table 1). Total number of oviposition events, total number of eggs produced per individual and the number of days from last oviposition to death were significantly correlated with lifespan (Table 1). Of the 66 individuals that completed one or more oviposition events, 45 died within 8 days and 50 within 11 days of the last oviposition event.

Oviposition characteristics

Clutch size variation was apparent both in the overall dataset and between individual tardigrades (Table 2). Of the 508 single clutches recorded, 71.5% comprised three to six eggs [three eggs $\times 97$ (19%); four eggs $\times 89$ (17%); five eggs $\times 92$ (18%); six eggs $\times 85$ (17%)]. Although the overall mean oviposition interval of individuals was 9.3 days (Table 2), 79% of single intervals were between 5 and 8 days amongst the total of 442 intervals recorded. Oviposition interval showed a significant positive correlation with lifespan (Table 2). Both hatching time and success were consistent whether considering eggs produced by individual tardigrades or the overall dataset (Table 2). Oviposition interval and hatching time showed significant negative correlations with clutch size while no significant correlation was identified between hatching success and clutch size (Table 2). Amongst the total of 508 oviposition events recorded, 39 included one or more eggs that did not hatch. Ten of these were produced in the relevant individual's final oviposition event that took place between days 52 and 108, and four individuals had failure eggs in their final two oviposition events.

Fig. 1 Survival over time for *Acutuncus antarcticus* under the constant culture conditions applied

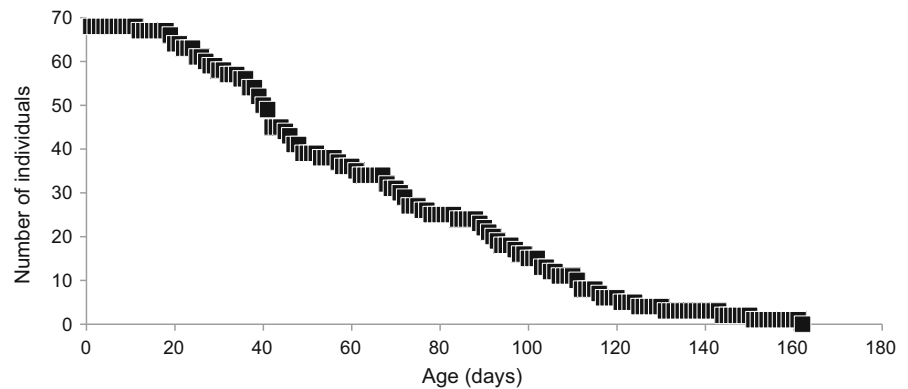


Table 1 Lifespan and lifetime reproductive traits of *Acutuncus antarcticus* under constant culture conditions at 15°C

	Lifespan (days)	Number of oviposition events	Number of eggs per individual	Age at first oviposition (days)	Days from last oviposition to death
Sample size	68	68	68	66	66
Mean \pm SD	69.2 ^a \pm 36.4 ^a	7.5 ^a \pm 4.3	34.4 ^a \pm 22.6 ^a	9.3 ^a \pm 1.1 ^a	9.7 \pm 10.0
Minimum–maximum	12–162 ^a	0–17	0–97	6–11	1–54
Median	65	7	32	10	7
Correlation with lifespan	–	$P < 0.001$	$P < 0.001$	$P = 0.14$	$P = 0.003$
Kendall's coefficient	–	0.81	0.66	–0.14	0.26

Correlations of each reproductive trait of individuals with lifespan were analysed using Kendall's tau statistic

^a Data reported in Tsujimoto et al. (2015)

Reproduction with age

Clutch size increased rapidly until 30 days and decreased slightly after 70 days (Fig. 3a). A slight increase in oviposition interval with age was evident (Fig. 3b). Hatching success decreased very slightly with advancing age (Fig. 3c). Of the 16 individuals that survived for more than 100 days, nine produced eggs with a 100% hatching success throughout their lifetime. Hatching time was virtually constant throughout the 161 days of the study period (Fig. 3d).

Discussion

Lifespan of *A. antarcticus* varied greatly among the individuals examined, as has been noted in studies of

other tardigrade species (Suzuki, 2003; Horikawa et al., 2008; Schill, 2013; Altiero et al., 2015). Considerable variation in lifespan between individuals within isogenic populations under constant laboratory conditions has also been observed in a model animal of ageing studies, the nematode *Caenorhabditis elegans* (Kirkwood & Finch, 2002), and also in the monogonont rotifers *Asplanchna brightwelli* and *Brachionus manjavacas* (Snell & King, 1977; Snell et al., 2012).

Clutch size of *A. antarcticus* in relation to age showed a rapid increase from the first oviposition until 30 days. An increase in clutch size early in the reproductive period was also observed in most individuals of the eutardigrade, *Milnesium* sp., a carnivorous species fed rotifers under experimental laboratory conditions (Suzuki, 2003). Increases in

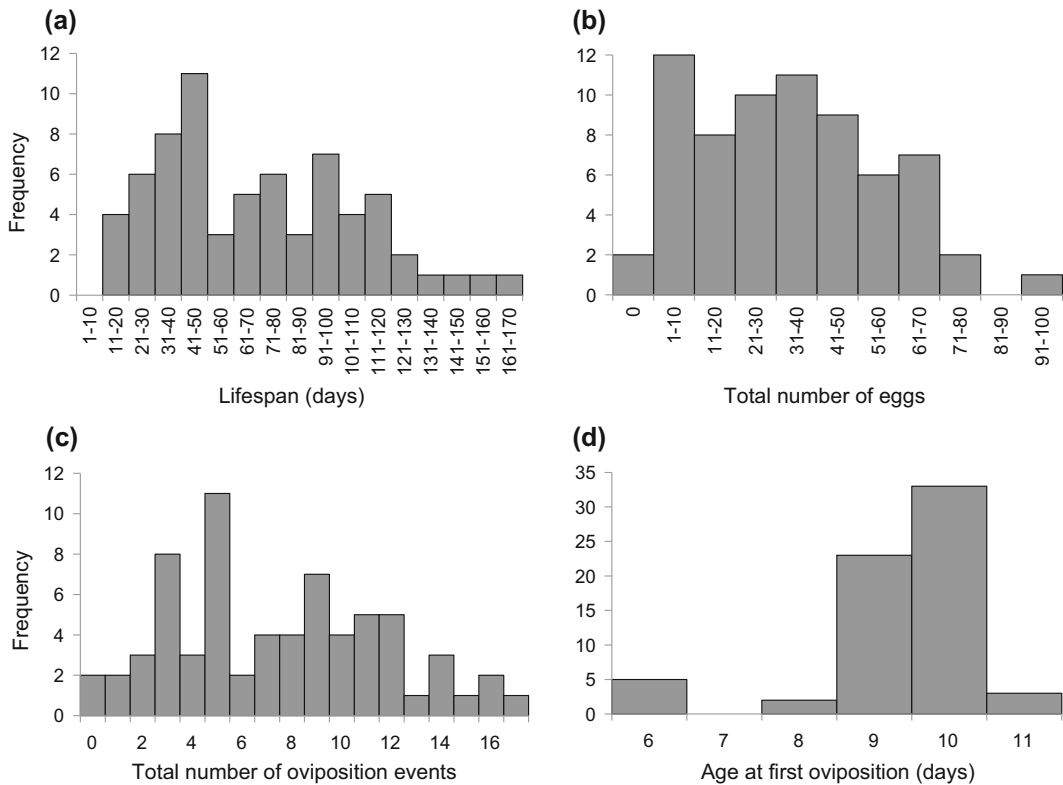


Fig. 2 Frequency distribution of lifespan (days) (a), total number of eggs (b), total number of oviposition events (c) and age at first oviposition (days) (d)

clutch size from the first oviposition to the second oviposition of *Milnesium* sp. occurred during the last stage of their body growth. Large variation in body size of individuals developing eggs was present in a natural population of another eutardigrade species *Pseudobiotus kathmanae* (Kathman & Nelson, 1987). A positive correlation between the body length of females and the number of oocytes was reported in three species of eutardigrades in a natural population field study of *Macrobiotus hufelandi*, *Paramacrobiotus richtersi* and *Diphascion pingue* in a moss carpet (Schuster & Greven, 2013). While no body size data were recorded in our study, a rapid increase in clutch size of *A. antarcticus* at the beginning of the reproductive period might also suggest that it is a relationship with the growth phase of animals. Weak effects of age were observed on oviposition interval and hatching success. While oviposition interval of *A. antarcticus* showed a small inverse relationship with the fluctuation in clutch size, it increased slightly with age. Hatching success decreased slightly although

remaining consistently high throughout the 161 days. While minor negative correlation with clutch size was detected, hatching time remained virtually constant throughout the 161-day period.

In the present study, the numbers of oviposition events and eggs produced per individual varied greatly and were correlated with lifespan. Clutch size fluctuated conspicuously, and oviposition interval increased slightly with age. However, the majority (71.5%) of individual clutches contained three to six eggs, and 79% of the single inter-oviposition intervals were between 5 and 8 days. Most individuals continued reproducing throughout their entire lifespan, and lifespan exerted a stronger effect than specific age on the total number of oviposition events completed and eggs produced per individual. Thus, variation in lifespan was reflected in the considerable variation in the lifetime numbers of oviposition events and eggs. Accordingly, these distributions were right-skewed. Although a significant positive correlation between inter-oviposition interval and lifespan was observed,

Table 2 Aspects of oviposition of *Acutuncus antarcticus* under constant culture conditions at 15°C

	Clutch size	Oviposition interval (days)	Hatching time (days)	Hatching success (%)
Lifetime averages for individual tardigrades				
Sample size	66	61 ^b	66	66
Mean ± SD	4.4 ± 1.2	9.3 ± 1.1	8.4 ± 0.3	97.5 ± 4.3
Minimum–maximum	1.4–6.5	5.0–11.0	7.6–9.0	85.7–100
Median	4.6	10	8.5	100
Correlation with lifespan	$P = 0.15$	$P < 0.001$	$P = 0.11$	$P = 0.13$
Kendall's coefficient	0.12	0.43	0.14	−0.14
Overall oviposition events				
Sample size	508	442 ^c	2,286	2,342
Mean ± SD	4.6 ± 1.8	7.7 ± 3.3	8.5 ^a ± 0.7 ^a	97.6 ^a
Minimum–maximum	1 ^a –10 ^a	3 ^a –32	7–12	–
Median (percentiles 25, 75)	5 (3, 6)	7 (6, 8)	8 (8, 9)	–
Correlation with clutch size	–	$P < 0.001$	$P < 0.001$	$P = 0.20$
Kendall's coefficient	–	−0.32	−0.12	0.049

Correlations of each reproductive trait of individuals with lifespan and traits of overall oviposition events with clutch size were analysed using Kendall's tau statistic

^a Data reported in Tsujimoto et al. (2015)

^b Data from individuals undergoing more than three oviposition events were included in these analysis in order to obtain reasonable estimation of the average

^c Data from clutches from the second oviposition event onwards were analysed. Since the days to the first oviposition include time of growth and do not represent a true oviposition interval, we calculated the intervals beginning from the days between the first and second oviposition events

no significant relationships between clutch size, hatching time or hatching success and lifespan were detected. While the hatching time of *A. antarcticus* was very stable across individuals in the current study, observations of a different population of the same species using a different rearing method indicate the possibility of phenotypic plasticity over generations in some of the reproductive parameters examined here including hatching time and success (Altiero et al., 2015). High variability in hatching time has also been reported within clones of the eutardigrade *Macrobio- tus richtersi* (Altiero et al., 2006, 2010).

In the population of parthenogenetic tardigrade *A. antarcticus* studied here, while the duration of the period from last oviposition to death increased slightly with lifespan, the majority of the individuals continued oviposition until shortly before death, with no suggestion of a post-reproductive lifespan which is typical in mammals and also in some invertebrate groups (Cohen, 2004; Jones et al., 2014). Similarly, individuals of another tardigrade, *Milnesium* sp., also reproduced continuously throughout their life (Suzuki, 2003). The slight decrease in clutch size of *A.*

antarcticus observed here after 70 days might suggest a small decline in fecundity after the mean lifespan at 69.2 days, but overall hatching success remained very high throughout the 161-day study period, decreasing very slightly. A major decrease of hatching success was observed between 50 and 110 days, when ten individuals produced one or two clutches including one or more eggs that did not hatch shortly before their death. However, more than half of the individuals that lived for more than 100 days produced egg batches with 100% hatching success throughout their lifespan and, as a result, hatching success was not significantly correlated with lifespan. Moreover, there was no effect of age or lifespan on hatching time. Together, these observations suggest that any decline in fertility with age in this species of tardigrade is minimal.

Senescence is usually indicated by age-related declines in survival and reproduction in association with progressive physiological deterioration (Kirkwood & Austad, 2000). Senescence is well documented in a variety of animals including wild populations of birds and mammals (Nussey et al., 2013). Among invertebrate species, the ageing process

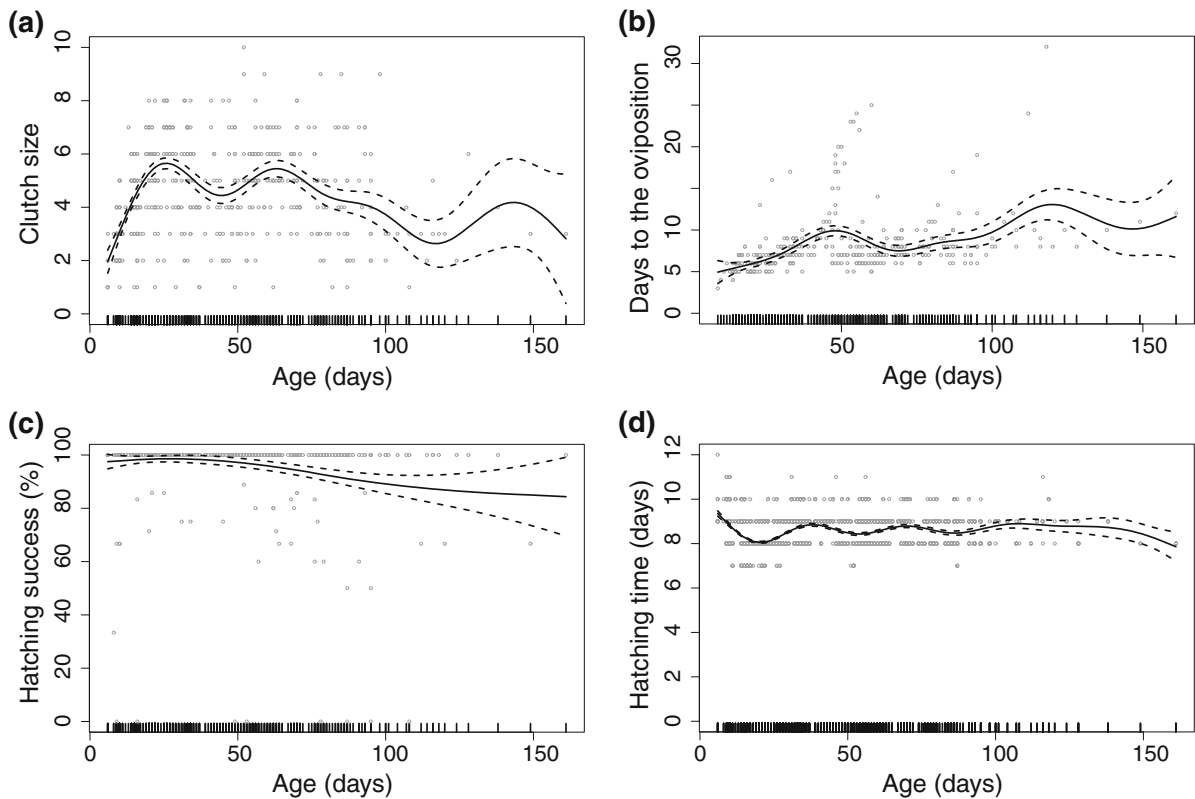


Fig. 3 Generalized additive mixed models (GAMMs) with thin plate regression spline for estimation of clutch size (a), oviposition interval (days) (b), hatching success (%) (c) and hatching time (days) (d). Dashed lines represent 95% confidence limits

in model species such as the nematode *C. elegans* and the fruit fly *Drosophila melanogaster* is the most studied (Dillin et al., 2002; Herndon et al., 2002; Giannakou et al., 2004). Reproductive senescence has been reported in both species, where rapid declines in fecundity begin at young to middle age due to the limitation of the fixed store of sperm in hermaphrodite *C. elegans* and apoptosis of ageing egg chambers in *D. melanogaster* (Hughes et al., 2007; Zhao et al., 2008). Fecundity peaks at very early ages followed by rapid declines have also been observed in the parthenogenetic bdelloid rotifers *Adineta ricciae* and *Macrotrachela quadricornifera* (Ricci et al., 1987; Ricci & Covino, 2005). In rotifers, the maximum potential fecundity of a female is fixed by the number of oocytes in her germarium (Gilbert, 1993). Decline in oocyte number is considered a major determinant of reproductive senescence in female mammals (Cohen, 2004). In the parthenogenetic tardigrade *A. antarcticus*, no information is available on oocyte numbers, but it would appear that neither the number of oocytes

nor the ageing of the reproductive organs restricts the continued successful reproduction of this species throughout its lifespan. The pattern of reproductive performance as a function of age in *A. antarcticus* is similar to those of the parthenogenetic water flea *Daphnia longispina* and the sexually reproducing human louse *Pediculus humanus* (see Jones et al., 2014).

Acutuncus antarcticus is known to be widespread in both terrestrial and freshwater habitats in Antarctica (McInnes, 1995; Gibson et al., 2007; Tsujimoto et al., 2014; Velasco-Castrillón et al., 2014). The natural environment in Antarctica is considered the most extreme and variable on Earth in terms of low temperature, limited water availability and short growing season (Smith, 1988; Peck et al., 2006). Asexual reproduction is considered an ancestral feature present in many of the major groups of invertebrates that are components of ecosystems in more extreme environments including Antarctica (Convey, 2010). Life cycle extension is widely used

by many taxa to survive in low-energy and ‘adversity selected’ environments such as those of Antarctica in order to overcome insufficient time to complete a full reproductive cycle within one season (Convey, 1996; Block et al., 2009). While prolonged survival has been hypothesised to be sacrificed for higher reproduction at early ages in some unfavourable environments (Kirkwood & Rose, 1991), prolonged maintenance of reproductive ability also gives an advantage in extended life cycles in the natural environment of Antarctica. In addition to the possession of parthenogenesis and anhydrobiosis, which are known to be important contributors to successful establishment in unstable environments for some micro-invertebrates including tardigrades (Bertolani, 2001) and bdelloid rotifers (Ricci, 1987), the minimal reproductive senescence of *A. antarcticus* revealed in our study might also be an important factor contributing to the successful establishment and widespread distribution of this species within the Antarctic region.

The in-depth analyses described here of reproductive traits collected from daily observations of *A. antarcticus* under constant laboratory conditions demonstrated, for the first time, the effect of lifespan and age on reproductive performance in a member of the phylum Tardigrada. Knowledge of the relationship between fertility and age, and of changes in reproductive characteristics over the lifespan, will improve our understanding of reproductive performance and population dynamics of tardigrades in the natural environment. While reproductive senescence has been observed in other groups of animals, no clear evidence was found in this tardigrade species, providing new insight into the reproductive biology and ageing of these invertebrates.

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