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Reproductive performance of the Antarctic tardigrades, *Acutuncus antarcticus* (Eutardigrada: Hypsibiidae), revived after being frozen for over 30 years and of their offspring

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Studies on the long-term survival of animals often focus on the specific instance of survival of animals only, and descriptions of subsequent reproduction are generally not reported. In this study, we recorded the reproductive performance of the first-generation offspring of the resuscitated individual (SB-1) and the hatchling of the resuscitated egg (SB-3) of the Antarctic tardigrade, *Acutuncus antarcticus*, after being frozen for 30.5 years. By providing further detailed description of the reproduction of SB-1 and SB-3 after revival, and then comparing the reproductive performance with that of their first-generation offspring, the possible indications of the damage accrued during the long-term preservation in SB-1 and SB-3 were more specifically detected. Additionally, the DNA analysis revealed two distinctively different mitochondrial genetic sequences of *A. antarcticus* between the SB strains and the LSW strain. The observed differences in some of the reproductive parameters between the two genetic types suggested a possible relationship between the life-history traits and genetic type in the species *A. antarcticus*. Further experiments using the SB-1 and SB-3 strains reared for a long period to exclude the instant effect of preservation are expected to improve our understanding of the mechanisms underlying the long-term survival of animals.

ADDITIONAL KEYWORDS: cryobiosis – cryptobiosis – freezing – long-term survival – reproduction.

INTRODUCTION

Long-term survival of micrometazoans, including tardigrades, has been one of the most studied of the extraordinary physiological characteristics of those animals (e.g. Guidetti & Jönsson, 2002). Meanwhile, there has been a debate on the uncertainty of the length of the long-term survival of animals reported in some early literature due to ambiguity of the methods or materials used (Jönsson & Bertolani, 2001; Fontaneto *et al.*, 2012). More recent studies, using samples stored at herbaria or freezers for certain recorded periods, provide precise information regarding length and means of storage, and thus accurate lengths of the long-term survival of studied animals (Guidetti & Jönsson, 2002; Newsham *et al.*, 2006; Jørgensen *et al.*, 2007; Kagoshima *et al.*, 2012). Studies on the long-term survival of animals have often focused on the specific instance of the survival of animals only and few studies have reported evidence of reproduction of animals after revival (Aroian *et al.*, 1993; Kagoshima *et al.*, 2012). Moreover, descriptions of subsequent reproduction are generally not reported.

In a previous study, we documented recovery conditions and reproduction immediately following the revival of the Antarctic tardigrades, *Acutuncus antarcticus* (Richters, 1904) (Eutardigrada:

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Hypsibiidae), retrieved from a frozen moss sample collected in Antarctica in 1983 and stored at –20 °C for 30.5 years (Tsujimoto *et al.*, 2016a). However, this previous paper focused on the specific instance of the revival and thus the detailed reproductive performance of the resuscitated individual and egg was not fully described. Furthermore, with data only on the reproduction of the resuscitated individuals, we were not able to discuss the possible effect of the long-term preservation of animals on reproduction following revival in greater detail.

Acutuncus antarcticus is known to be one of the most widespread Antarctic tardigrade species (Velasco-Castrillón *et al.*, 2014). This species is endemic to Antarctica and has been collected from various types of substrates, such as soil, moss, algae, lichen and phytobenthos, in both terrestrial and freshwater environments in Antarctica (Dastych, 1991; McInnes, 1995; Gibson *et al.*, 2007; Tsujimoto *et al.*, 2014). Recent molecular studies revealed high genetic diversity within *A. antarcticus* and the presence of two distinct genetic types in the Antarctic continent (Czechowski *et al.*, 2012; Velasco-Castrillón *et al.*, 2015; Cesari *et al.*, 2016). Moreover, life-history traits of *A. antarcticus* have also been described using individually established strains (Altiero *et al.*, 2015; Tsujimoto *et al.*, 2015). However, the analysed life-history traits were reported independently of the DNA analysis, and their specific life-history traits in relation to the patterns of the genetic types of *A. antarcticus* have not been evaluated. Consequently, it is unclear if various strains of *A. antarcticus* studied previously and reared under identical laboratory conditions (Tsujimoto *et al.*, 2015, 2016a, 2016b) exhibit similar reproductive performance.

In the current study, we report detailed reproduction of the first-generation offspring of the resuscitated individual, SB-1, and the hatchling of the resuscitated egg, SB-3, with additional descriptions of the subsequent reproduction of SB-1 and SB-3 themselves after revival. By comparing the reproductive performance of SB-1, SB-3 and their offspring, we identified possible indications of damage and recovery in the revived animals, which enhances our understanding of the mechanisms underlying the long-term survival of animals. Furthermore, we conducted DNA analysis of the SB-1 and SB-3 strains, in addition to the previously established strain of *A. antarcticus* (LSW) with their life-history traits having been reported (Tsujimoto *et al.*, 2015, 2016b), to determine the possible relationships between the life-history traits and genetic types of this species.

MATERIAL AND METHODS

Sample collection and culture conditions

This study uses the revived individual SB-1 and the hatchling from the revived egg, SB-3, and their first offspring of the eutardigrade *Acutuncus antarcticus* recovered from a moss sample collected in Yukidori Valley, Langhovde, Sôya coast, Dronning Maud Land (East Antarctica; 69°14′30″S, 39°46′00″E) in November 1983 during the 24th Japanese Antarctic Research Expedition (JARE) winter operation (Tsujimoto *et al.*, 2016a). The moss sample of *Bryum argenteum* Hedw., F01096 (NIPR), was stored at –20 °C before being thawed in May 2014. The revived tardigrade, SB-1, and the egg, SB-3, were retrieved using a pipette under a dissection microscope. SB-1, SB-3 and their first-generation offspring were reared in individual wells on culture plates in the dark at 15 °C. The TPP® tissue culture plate (12 wells, flat bottom) was prepared with a layer of 300 µL of 1.5% agar gel on the bottom of each well, to which 600 µL of Volvic® water and 1.8 µL of a suspension of *Chlorella* sp. (Chlorella Industry Co., Japan) was added as a food source (see: Tsujimoto *et al.*, 2015).

Reproductive performance

The studied strains of *Acutuncus antarcticus* are parthenogenetic and males were never observed during the experiments. The individual tardigrades were inspected daily and their reproduction was monitored. Animals were transferred to new culture dishes every week (following Tsujimoto *et al.*, 2015). Eggs were laid freely from the exuvia and new clutches were isolated on the day of oviposition, separated and individually transferred to wells on new culture plates. Subsequent hatching of the isolated eggs was monitored daily until 30 days after oviposition. Data on timing and clutch size of each oviposition event, egg development time to hatching (hatching time) and hatching success were recorded. Because SB-1 was accidentally dried during transfer after its fifth oviposition, no records were taken after this event for this individual. Observations were made using a dissecting microscope (Olympus SZX7) at 56× magnification. Additionally, Kruskal–Wallis rank sum test was conducted using R v.3.5.1 to determine if there were any significant differences between the averages of lifespan, age at first oviposition and hatching success for the individual data, and overall clutch size, oviposition intervals and hatching time in the three strains, LSW strain and the first-generation offspring of SB-1 and SB-3 (SB-1 F1s and SB-3 F1s, respectively). Furthermore, pairwise comparisons using Wilcoxon rank sum test were

conducted (R v.3.5.1) to find out which pairs of strains are significantly different in the parameters that had been found significantly different in Kruskal–Wallis rank sum test.

DNA sequence analysis

In addition to the two strains obtained from the frozen moss in the current study (SB-1 and SB-3), a strain of *A. antarcticus* collected from a freshwater lake (Hamagiku-Ike Lake in Skarvsnes; 69°29′26″S 39°36′05″E) in Dronning Maud Land (Tsujimoto *et al.*, 2014), LSW (after Little Snow White), was used for DNA sequence analysis. This strain was reared from an individual of *A. antarcticus* retrieved from the phytobenthos sample frozen at –70 °C for five years, and its reproductive characteristics were recorded in detail after being reared under the same laboratory conditions as the SB strains (Tsujimoto *et al.*, 2015, 2016b). Genomic DNA was prepared from individual samples of each strain by the alkaline lysis method (Stanton *et al.*, 1998). DNA fragments of the 18S ribosomal RNA gene locus (18S rDNA) and mitochondrial cytochrome *c* oxidase subunit 1 (*cox1*) locus were amplified by polymerase chain reaction (PCR), using SSU04F (GCTTGTCTCAAAGATTAAGCC) and SSU81R (TGATCC(A/T)(T/G)C(C/T)GCAGGTTCAC) for the 18S rDNA locus (Blaxter *et al.*, 1998), and LCO1490 (GGTCAACAAATCATAAGATATTGG) and HCO2198 (TAAACTTCAGGGTGACCAAAAAATCA) for the *cox1* locus (Sands *et al.*, 2008). PCR conditions were: 94 °C for 2 min, followed by 40 cycles at 94 °C for 10 s, 52 °C for 30 s and 72 °C for 1 min, and 72 °C for 10 min (Kagoshima *et al.*, 2013). PCR products were separated by agarose gel electrophoresis and purified using QIAquick Gel Extraction kit (Qiagen, USA). Sequencing reactions were performed with Big-Dye terminator cycle sequencing kits (Applied Biosystems, USA). Sequences were assembled and compared with published sequences in GenBank by BLAST search (Altschul *et al.*, 1997).

RESULTS

Lifespan and reproductive performance of SB-1 F1 and SB-3 F1

The lifespans of the 14 individuals of SB-1 F1s ranges from 28 to 78 days (mean ± SD, 48.0 ± 16.1 days) (Table 1). The first oviposition occurs at the age of 6 to 12 days. The average number (±SD) of oviposition events and eggs per individual were 5.8 (±3.2) and 21.6 (±13.8), respectively. Average (±SD) clutch size and hatching time were 3.5 (±0.8) and 8.8 (±0.4) days, respectively. Hatching success ranged from 0 to 100% (mean ± SD, 83.6 ± 26.5%; median, 94.6%). Oviposition intervals from the first oviposition to the fifth ovipositoin of SB-1 were 4, 5, 4 and 9 days, respectively (average ± SD, 5.5 ± 2.4 days; median, 4.5 days) (Table 2). Clutch sizes from the first oviposition to the fifth oviposition of SB-1 were 3, 4, 4, 5, and 3 with 1, 4, 4, 5, and 0 eggs hatched, respectively (Table 2). Clutch size and hatching success of SB-1 were within the range of those of its first-generation offspring SB-1 F1s. The last oviposition interval of SB-1, which was 9 days, went over the range of overall oviposition intervals of SB-1 F1s (minimum of 3 days and maximum of 6 days) (Tables 2, 3). The hatching time of the egg deposited at the first oviposition by SB-1, which was 19 days, went over the range of overall hatching time of the eggs deposited by SB-1 F1s (minimum of 7 days and maximum of 13 days; Tables 2, 3).

The lifespans of the seven individuals of SB-3 F1s ranged from 32 to 64 days (mean ± SD, 47.4 ± 12.6 days) (Table 1). The first oviposition occurred at the age of 7 to 10 days. The average (±SD) number of oviposition events and eggs per individual were 6.3 (±2.8) and 28.9 (±14.9), respectively. The average (±SD) clutch size and hatching time were 4.3 (±1.0) and 9.5 (±0.3) days, respectively. The average (±SD) clutch size of the six individuals of SB-3 F1s with four or more oviposition events from the first to the fourth ovipositions were 2.3 (±0.5), 3.9 (±0.9), 5.3 (±1.0) and 6.3 (±0.8). Hatching success ranged from 60.0 to 88.9% (mean ± SD, 73.4 ± 11.9%; median, 73.8%). Oviposition intervals from the first oviposition to the sixth oviposition of SB-3 were 6, 5, 4, 4 and 5 days, respectively (mean ± SD, 4.8 ± 0.8 days; median, 5 days; Table 2). Clutch size from the first oviposition to the sixth oviposition of SB-3 were 1, 2, 4, 1, 3 and 4, with 0, 1, 4, 0, 0 and 2 eggs hatched, respectively (Table 2). Hatching success of SB-3 (46.7%) went below the range of hatching success of SB-3 F1s (minimum of 60.0% and maximum of 88.9%) and the clutch size of one-third of the ovipositions in SB-3 (which was 1) went below the range of overall clutch size of SB-3 F1s (minimum of 2 and maximum of 7; Tables 2, 3).

Reproductive characteristics and lifespan of SB-1, SB-3 and LSW strains

Hatching time showed little variability within both SB-1 F1s and SB-3 F1s and was fairly consistent whether considering eggs produced by individual tardigrades or the overall dataset (Tables 1, 3). There was no significant difference detected in lifespan and age at first oviposition among the three strains (Table 4). Meanwhile, there were highly significant differences in overall hatching time among the three strains ($P < 0.001$ in all three combinations) (Tables 3, 4; Fig. 1). Hatching success of the eggs deposited by SB-1 and SB-3 strains varied compared with that of the LSW strain (Table 1), and was significantly lower

Table 1. Lifespan and reproductive performance of *Acutuncus antarcticus* SB-1, SB-3 and their first-generation offspring under constant culture conditions at 15 °C

	Oviposition number	Lifespan (d)	Hatching time (d)	Age at 1st oviposition (d)	Number of oviposition events	Number of eggs per individual	Average clutch size	Average oviposition intervals (d)	Average hatching time (d)	Hatching success (%)	Reference
SB-1		-	-	-	5[a]	19[a]	3.8	5.5	10.4	73.7	Tsujimoto *et al.*, 2016a
SB-3		33	-	8	6	15	2.5	4.8	9.9	46.7	Tsujimoto *et al.*, 2016a
SB-1 F1	1	78	19	6	11	38	3.5	4.6	8.8	94.7	
	2	55	11	10	8	33	4.1	4.3	9.0	100	
	2	43	11	9	6	21	3.5	4.2	9.3	66.7	
	2	30	13	8	2	5	2.5	-	8.8	80.0	
	2	67	13	8	10	36	3.6	4.7	8.4	94.4	
	3	42	8	9	6	28	4.7	4.4	8.3	96.4	
	3	58	9	8	9	40	4.4	4.5	8.3	90.0	
	3	30	10	8	3	12	4.0	4.0	8.3	75.0	
	3	35	10	7	2	7	3.5	-	8.6	100	
	4	28	8	12	2	4	2.0	-	-	0.0	
	4	65	8	11	9	40	4.4	4.4	9.2	97.5	
	4	42	8	12	6	18	3.0	4.2	9.3	100	
	4	36	9	11	4	13	3.3	4.0	9.1	100	
	4	63	8	11	3	8	2.7	4.5	8.5	75.0	
	Mean ± SD	48.0 ± 16.1	10.4 ± 3.1	9.3 ± 1.9	5.8 ± 3.2	21.6 ± 13.8	3.5 ± 0.8	4.3 ± 0.2	8.8 ± 0.4	83.6 ± 26.5	
	Median	42.5	9.5	9	6	19.5	3.5	4.4	8.8	94.6	
SB-3 F1	2	32	11	9	2	5	2.5	-	9.3	60.0	
	3	56	9	9	8	42	5.3	4.7	9.2	73.8	
	3	47	9	8	7	38	5.4	4.7	9.5	84.2	
	3	32	10	7	4	18	4.5	4.3	9.6	88.9	
	3	64	10	7	10	46	4.6	4.9	10.0	65.2	
	6	43	9	10	5	20	4.0	4.8	9.3	60.0	
	6	58	11	8	8	33	4.1	4.7	9.3	81.8	
	Mean ± SD	47.4 ± 12.6	9.9 ± 0.9	8.3 ± 1.1	6.3 ± 2.8	28.9 ± 14.9	4.3 ± 1.0	4.7 ± 0.2	9.5 ± 0.3	73.4 ± 11.9	
	Median	47	10	8	7	33	4.5	4.7	9.3	73.8	
LSW	Mean ± SD	69.2 ± 36.4	-	9.3 ± 1.1	7.5 ± 4.3	34.4 ± 22.6	4.4 ± 1.2	7.4 [b] ± 1.5 [b]	8.5 [b] ± 0.3	97.5 ± 4.3	Tsujimoto *et al.*, 2015, 2016b
	Median (min–max)	65 (12–162)	-	10 (6–11)	7 (0–17)	32 (0–97)	4.6 (1.4–6.5)	7.0 [b] (5.0–11.5 [b])	8.5 (7.6–9.0)	100 (80.0 [b]–100)	Tsujimoto *et al.*, 2016b
	Sample size	68	-	66	68	68	66	61[c]	66	66	

[a] Not lifetime data.
[b] Updated and corrected from the data originally presented in Tsujimoto *et al.*, 2016b.
[c] Data from individuals undergoing three or more oviposition events were included in these analysis in order to obtain reasonable estimation of the average.

Table 2. Reproduction of *Acutuncus antarcticus* SB-1 and SB-3 after rehydration and being thawed from 30.5 years of being frozen

Individual	Oviposition sequence	Days after rehydration	Oviposition interval (d)	Clutch size	Number of eggs hatched	Hatching time (d)
SB-1	1	23		3	1	19
	2	27	4	4	4	11, 11, 13, 13
	3	32	5	4	4	8, 9, 10, 10
	4	36	4	5	5	8, 8, 8, 8, 9
	5	45	9	3	0	
SB-3	1	14 (8)		1	0	
	2	20 (14)	6	2	1	11
	3	25 (19)	5	4	4	9, 9, 10, 10
	4	29 (23)	4	1	0	
	5	33 (27)	4	3	0	
	6	38 (32)	5	4	2	9, 11

Numbers in the parentheses indicate the age in days after the birth of SB-3.

Table 3. Aspects of oviposition of *Acutuncus antarcticus* SB-1, SB-3 and LSW strains under constant culture conditions at 15 °C

		SB-1 F1	SB-3 F1	LSW (Tsujimoto *et al.*, 2016b)
Clutch size	Sample size	81	44	508
	Mean ± SD	3.7 ± 1.3	4.6 ± 1.7	4.6 ± 1.8
	Min–max	1–7	2–7	1–10
	Median (percentiles 25, 75)	4 (3, 5)	4.5 (3, 6)	5 (3, 6)
Oviposition interval (d)	Sample size	67	37	442
	Mean ± SD	4.4 ± 0.6	4.7 ± 0.9	7.7 ± 3.3
	Min–max	3–6	3–6	3–32
	Median (percentiles 25, 75)	4 (4, 5)	5 (4, 5)	7 (6, 8)
Hatching time (d)	Sample size	276	144	2286
	Mean ± SD	8.8 ± 0.9	9.5 ± 1.1	8.5 ± 0.7
	Min–max	7–13	8–15	7–12
	Median (percentiles 25, 75)	9 (8, 9)	9 (9, 10)	8 (8, 9)

Table 4. Results of the Kruskal–Wallis rank sum test and the Wilcoxon rank sum test performed for the life-history traits of *Acutuncus antarcticus* SB-1 F1, SB-3 F1 and LSW

	Parameter	Kruskal-Wallis rank sum test	Pairwise comparisons using Wilcoxon rank sum test		
			LSW-SB-1	LSW-SB-3	SB-1-SB-3
Individual tardigrades (Table 1)	Lifespan	0.091			
	Age at first oviposition	0.064			
	Hatching success	< 0.001	< 0.01	< 0.001	< 0.05
Overall oviposition events (Table 3)	Clutch size	< 0.001	< 0.001	0.999	< 0.05
	Oviposition interval	< 0.001	< 0.001	< 0.001	0.066
	Hatching time	< 0.001	< 0.001	< 0.001	< 0.001

Significant level: significant at $0.001 \leq P < 0.05$, highly significant at <0.001

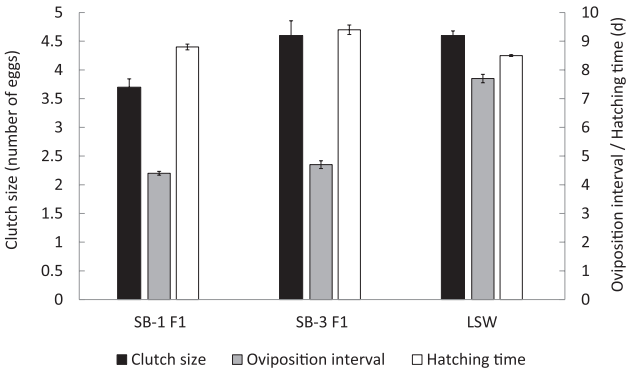

Figure 1. Mean clutch size (left axis), oviposition interval (right axis) and hatching time (right axis) of SB-1 F1, SB-3 F1 and LSW (compare to Tables 3 and 4). Vertical bars represent ± standard errors of the means.

than that of the LSW strain ($P < 0.01$ and $P < 0.001$, respectively) (Table 4). Overall clutch size of SB-1 F1s was significantly smaller than that of SB-3 F1s ($P < 0.05$) and LSW ($P < 0.001$), while there was no significant difference in clutch size between SB-3 F1 and LSW (Tables 3, 4; Fig. 1). Overall oviposition intervals of the LSW strain were significantly longer than those of SB-1 F1s ($P < 0.001$) and SB-3 F1s ($P < 0.001$), and there was no significant difference between SB-1 F1 and SB-3 F1 oviposition intervals (Tables 3, 4; Fig. 1).

DNA ANALYSES

Partial sequences of 18S rDNA and *cox1* gene from the three *A. antarcticus* strains (SB-1, SB-3 and LSW) were obtained. The three strains shared identical nuclear 18S rDNA sequences (GenBank accession nos. LC089867, LC089868 and LC089869, respectively) with other samples of *A. antarcticus* reported previously (e.g. GenBank accession nos. AB753790, EF632432). However, SB-1 and SB-3 had identical *cox1* gene sequences (accession nos. LC089870 and LC089871, respectively), which differed from that of LSW (accession no. LC089872) (Tsujimoto *et al.*, 2015, 2016b). LSW shared similar *cox1* gene sequences with the majority of the previously recorded *A. antarcticus* samples, including a strain obtained from a terrestrial moss sample (Kagoshima *et al.*, 2013) collected near the same location (accession no. AB753792) and the samples from some other areas in Antarctica (e.g. accession nos. JX865305 and KJ 856971). The similarity between the *cox1* gene DNA sequences of LSW compared to SB-1 and SB-3 was 81.8% (720 bp). Additionally, the SB-1 and SB-3 strains had almost identical *cox1* gene sequences to those of three *A. antarcticus* samples previously reported from Dronning Maud Land and the Queen Maud Mountains (a subdivision of the Transantarctic Mountains of central Antarctica) (accession nos. KJ856977, KJ856978 and KJ859679) (Velasco-Castrillón *et al.*, 2015).

DISCUSSION

Although hatching success of SB-1 F1s ranges from 0 to 100%, among the 14 individuals recorded, nine produced egg batches with more than 90.0% hatching success and four produced egg batches with 100% hatching success, giving the median of 94.6%. A median hatching success of 94.6% is considerably high relative to the numbers previously reported on tardigrades reared in the laboratories, including another strain of *Acutuncus antarcticus* reared in a different laboratory (see: Altiero *et al.*, 2015; Tsujimoto *et al.*, 2015). Oviposition intervals and hatching time were fairly consistent both among the individuals and overall ovipositions of SB-1 F1s. On the other hand, the interval to the last oviposition in SB-1 was double (9 days) that of the median (4.5 days) of the other four intervals. Likewise, a longer hatching time of the first egg was also recorded in SB-1 (Tsujimoto *et al.*, 2016a). This longer oviposition interval of SB-1 before the last oviposition might be also an indication of the possible damage accrued during the long-term preservation in the resuscitated individual as discussed previously (Tsujimoto *et al.*, 2016a). Although none of the three eggs deposited by SB-1 during the last oviposition hatched, the hatchling of the first egg, which required the exceptionally long hatching time of 19 days, lived for the longest amount of time (78 days) and demonstrated high reproductive performance (deposited a total of 38 eggs with 94.7% hatching success). Therefore, we hypothesize that the extensive damage may have been largely overcome during the extended development time and the repair activities may not have been continued after hatching.

Hatching success of SB-3 F1s ranged from 60.0 to 88.9%, with the mean and the median around 74%. Oviposition intervals and hatching time were also fairly consistent both among the individuals and overall ovipositions of SB-3 F1s, and those of SB-3 fell within the same range with SB-3 F1s. In the meantime, the average clutch size of, and the total number of eggs deposited by, SB-3 were smaller than those of the majority of SB-3 F1s. Previous studies demonstrated a rapid increase in clutch size at the beginning of the reproductive period in the *A. antarcticus* LSW strain and *Milnesium* sp. in possible relation to the growth phase of the animals (Suzuki, 2003; Tsujimoto *et al.*, 2016b). Similarly, the average clutch size of SB-3 F1s showed an increase from the first to fourth oviposition events. Taking

the increase of clutch size into account at early ages of tardigrades, the 15 eggs produced through six oviposition events by SB-3 is considered low, whereas there was an individual of SB-3 F1 that deposited five eggs in two oviposition events. In addition to the lower clutch size of SB-3, the hatching success of of SB-3 (46.7%) was also lower than that of all the individuals of SB-3 F1s studied. Damage during the embryonic stage was expressed in later stages in the life cycle in another species of tardigrade, *Hypsibius exemplaris* (former *H. dujardini*), and an apparent reduction in fertility was observed in the hatchlings of irradiated eggs (Beltrán-Pardo *et al.*, 2015). The lower fertility (number of eggs laid) and fecundity (hatching success of the eggs laid) of *A. antarcticus* SB-3, the hatchling of the resuscitated egg, compared with those of SB-3 F1s could be a possible indication of the residual damage from the embryonic stage.

It has been reported in several tardigrade species, including *H. exemplaris*, *Milnesium* cf. *tardigradum* and *Richtersius coronifer*, that eggs in early stages of development compared with late stages are more sensitive to environmental damage, such as desiccation, freezing and radiation, which often results in lower hatching success (Schill & Fritz, 2008; Hengherr *et al.*, 2010; Beltrán-Pardo *et al.*, 2013, 2015; Jönsson *et al.*, 2013). In addition, a delay in development was observed in the irradiated eggs of *M.* cf. *tardigradum* and this delay was explained by the time of DNA repair potentially taking place after receiving irradiation-induced damage (Beltrán-Pardo *et al.*, 2013). Although there were no statistical differences observed between early and late stages of development in hatching success and time of the eggs exposed to either desiccation or UV radiation using a different strain of *A. antarcticus* (Giovannini *et al.*, 2018), and the development stage of the SB-3 egg that was collected in Antarctica and later stored frozen for more than 30 years is not known, the lower fertility and fecundity of SB-3 suggest a possibility that the resuscitated egg was collected and preserved at an early stage of embryonic development.

The resuscitated individual (SB-1) and egg (SB-3) had a different genotype from the *A. antarcticus* LWS strain, whose life-history traits and characteristics in reproductive performance were studied in detail using the same protocol described by Tsujimoto *et al.* (2015, 2016b). The hatching success of the LSW strain in those studies was very high (97.5 ± 4.3%), with more than half of the individuals having 100% hatching success throughout their life (Tsujimoto *et al.*, 2015, 2016b). The hatching success of the SB-1 and SB-3 strains in the current study was lower than that of the LSW strain. In addition, oviposition intervals of SB strains were shorter than those of the LSW strain.

Whereas the *A. antarcticus* LSW genetic type is widely distributed in both terrestrial and freshwater environments throughout the Antarctic continent (Czechowski *et al.*, 2012; Velasco-Castrillón *et al.*, 2015; Cesari *et al.*, 2016), the SB genetic type has only been reported from terrestrial soil samples collected in two limited areas (Queen Maud Mountains and Dronning Maud Land) in Antarctica (Velasco-Castrillón *et al.*, 2015). Similarly, while SB-1 and SB-3 in our study were originally extracted from a terrestrial moss sample collected near Syowa Station located in Dronning Maud Land (Tsujimoto *et al.*, 2016a), the LSW strain and another strain of the *A. antarcticus* LSW genetic type were extracted, respectively, from a phytobenthos sample of a freshwater lake and a terrestrial moss sample collected near Syowa Station (Kagoshima *et al.*, 2013; Tsujimoto *et al.*, 2014, 2015).

Although further detailed examinations that exclude the effect of the long-term preservation are expected, our findings above suggest possible relationships between the distribution patterns or life-history traits and the *A. antarcticus* genetic types SB and LSW. Additionally, hatching time differed independently from each other among the three strains and clutch size of SB-1 F1s differed from that of LSW and SB-3 F1s. These findings indicate possible variability in reproductive performance within the same genetic type of *A. antarcticus* and/or presence of some possible permanent damage preserved within the SB strains due to long-term freezing, which can affect reproductive performance.

We recorded reproductive performance of the first-generation offspring (F1) of the resuscitated individual (SB-1) and the hatchling of the resuscitated egg (SB-3) of the Antarctic tardigrade, *A. antarcticus*, after being frozen for 30.5 years. By providing a further detailed description on the reproduction of SB-1 and SB-3 after revival, and then comparing the reproductive performance of SB-1 and SB-3 with that of their first-generation offspring (SB-1 F1s and SB-3 F1s), we further detected possible indications of the damage accrued during the long-term preservation of SB-1 and SB-3. Furthermore, the indication that SB-3 may have been collected at an early stage of embryonic development was suggested because it had lower fertility and fecundity than its offspring. Moreover, DNA analysis revealed two distinctively different mitochondrial genetic sequences of *A. antarcticus* between the SB and LSW strains. The differences in some of the reproductive parameters between the two strains observed in the current study indicates a possible relationship between *A. antarcticus* life-history traits and genetic types (SB and LSW). With the possible different distribution patterns of *A. antarcticus* SB and LSW genetic types reported within Antarctica, further understanding of the

life-history traits of each strain is required in order to enhance our knowledge regarding the life-history strategies of the Antarctic tardigrades and their distribution patterns within Antarctica. Moreover, further experiments using the SB-1 and SB-3 strains reared for a long period of time enough to exclude the instant effect of the long-term preservation of 30.5 years under a wide range of controlled conditions are expected to improve our understanding of the mechanisms underlying long-term survival of animals.

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