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Spermatozoa morphology changes during reproduction and first observation of acrosomal contact in two dioecious species of Macrobiotidae (Tardigrada: Eutardigrada)

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Summary

Mating behaviours for two species of dioecious eutardigrades: a strain of *Paramacrobiotus* sp. and *Macrobiotus shonaicus* (Stec *et al*., 2018) have been recorded previously, and observations have indicated that spermatozoa of both species are first released into the environment, then swim through the cloaca of the females and into the spermatheca. The fusion of gamete nuclei has not yet occurred in a laid egg. Therefore, it has been suggested that fertilization is completed externally as the egg is released into the environment before the nuclei of the gametes fuse. In the present study, the spermatozoa of both *Paramacrobiotus* sp. and *M. shonaicus* spermatozoa underwent morphological changes during reproduction. In morphometrical analyses of testicular spermatozoa, the tail, mid-piece, nucleus, and acrosome were significantly longer in *Paramacrobiotus* sp. compared with *M. shonaicus*. The nuclei of both the testicular and spermathecal spermatozoa were equally coiled, but the latter had shorter tails in both species. These spermatozoa were present on the surface of the egg chorion after oviposition. The tip of the acrosomes lay buried in the chorion, suggesting that penetration had occurred. We also proposed that the reduced tail is a conserved trait, at least in Macrobiotidae.

Introduction

Fertilization is an essential process in sexual reproduction in hermaphrodites as well as dioecious animals. In general, the fertilization process begins with the spermatozoa approaching the egg and is completed by fusion of both gametes.

Both hermaphroditism and dioecious reproduction have been reported in tardigrades (Bertolani, 2001). In culture, two species of hermaphroditic Eutardigrada – *Isohypsibius monoicus* Bertolani, 1981 and *Macrobiotus joannae* Pilato and Binda, 1983 – self-fertilized (see Altiero and Rebecchi, 2001). In contrast, mating behaviour has been recorded in three dioecious species of Eutardigrada: *Isohypsibius dastychi* (Pilato *et al*., 1982) (Hypsibiidae), which oviposits in the female exuviae (see Bingemer *et al*., 2016); and *Paramacrobiotus* sp. and *Macrobiotus shonaicus* (Stec *et al*., 2018) (Macrobiotidae), which lay their eggs freely (see Sugiura *et al*., 2019). The observations of the two Macrobiotidae species clearly indicated that spermatozoa are first released into the environment, then swim to the cloaca of a female. After mating, the female stores the spermatozoa in her spermatheca, which is close to the external opening of the cloaca (Altiero *et al*., 2018; Sugiura *et al*., 2019). Spermatheca have been found in females of many Macrobiotidae species. Furthermore, because the cloaca, ovary, and spermatheca are in close proximity, fertilization was believed to occur inside the female (Rebecchi, 1997; Bertolani, 2001; Bertolani and Rebecchi, 1999; Rebecchi and Guidi, 2000). However, a recent study showed that a spermatozoon nucleus entered the chorion of an egg, and the spermatozoon and egg nuclei were clearly distinguished inside the spawned egg, suggesting that fertilization was not completed in the female's body (Sugiura *et al*., 2019). Only one report has weakly supported external fertilization in a species in the *Paramacrobiotus richtersi* complex, which lays eggs freely, by observing a spermatozoon on the chorion of an egg (Guidetti *et al*., 2019), however the details of the events during the fertilization process in dioecious tardigrades are still unclear.

In our study we observed acrosomal contact between a spermatozoon and the surface of an egg's chorion in two species of dioecious Macrobiotidae – *Paramacrobiotus* sp. and *M. shonaicus* – and concluded: (a) that the two species significantly differ in spermatozoa morphometrics; and (b) morphology changes inside female. This process has been observed previously in another Macrobiotidae species, *Xerobiotus pseudohufelandi* Iharos, 1966 (see Rebecchi, 1997), and strongly suggests that this morphological change is conserved in Macrobiotidae.

Materials and methods

Tardigrade culture conditions

Paramacrobiotus sp. TYO strain and *M. shonaicus* were cultured using the methods described in Sugiura *et al.* (2019). Plastic dishes (AS ONE, Japan), either 30 mm or 90 mm diameter, with 1.2% agar gel (nacalai tesque, Japan) at the bottom were filled with Volvic water and maintained in the dark at 20°C. The rotifer *Lecane inermis* and green alga *Chlorella vulgaris* (Recenttec, Japan) were used as food. The water was changed twice each week, and the plastic dishes were replaced once each month. The tardigrades were observed under a stereomicroscope Mz.95 (Leica, Germany) or SZH10 (Olympus, Japan) and were photographed and videoed with an attachable camera TG-5 (Olympus).

Observations of mating in cultures

Mating observations in both species were performed in accordance with the methods described in Sugiura *et al.* (2019). Tardigrades of each species were kept in same-sex groups for at least 1 week before the mating experiment. Males and females were identified by the presence of testes or oocytes in their gonads and then cultured in separate dishes. Mating occurred in 30 mm culture dishes without a food source. Post-mating females were cultured individually and observed until they laid eggs.

Observations and morphometrics of gametes

Testicular and spermathecal spermatozoa were obtained from males and mated females of both species. To observe them using phase-contrast and fluorescent microscopy, individual tardigrades were placed on poly-L-lysine coated slides, then dissected with a 26G needle under a stereomicroscope. The sample was fixed in 2.5% glutaraldehyde/phosphate-buffered saline (PBS) for 1 h. After the samples were briefly washed three times with PBS, nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI) for 10 min. Samples were again washed three times with PBS, and slides were mounted in Fluoro-KEEPER Antifade Reagent Non-Hardening Type (nacalai tesque). Observations were made using an Axio Imager M1 microscope (Carl ZEISS, Germany). For each of 50 testicular spermatozoa from eight males of each species, four structures (tail, mid-piece, DAPI-stained nucleus and acrosome; Rebecchi, 1997) were measured using ImageJ software (https://imagej.nih.gov/ij). The length of tail tuft was excluded because of technical difficulties.

More than 1 day after being laid, 30 eggs from cultures of *Paramacrobiotus* sp. were placed in a droplet of Hoyer's medium onto microscope slides and secured with coverslips. The slides were dried at 60°C for 5 days and sealed with transparent nail polish. Eggs were examined under phase-contrast and differential interference contrast microscopy using an Axio Imager M1. Bare diameter, full diameter, process height, process base width, inter-process distance, process base/width ratio and number of processes were measured in accordance with the methods described in Kaczmarek and Michalczyk (2017) and Stec *et al.* (2018, 2020). All measurements were obtained using ImageJ software and are presented as μm.

Morphometric data on eggs from *M. shonaicus* were obtained from Sugiura *et al.* (2020; labelled 'SHONAI' in Supplementary

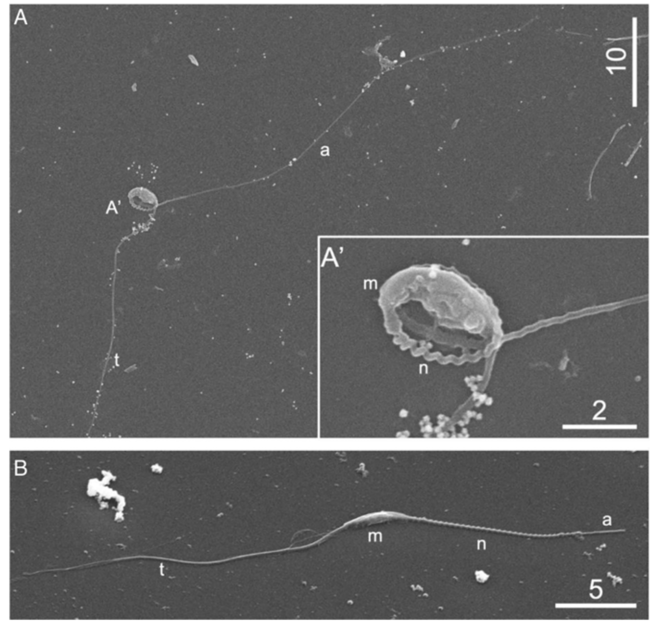

Figure 1. Testicular spermatozoa of *Paramacrobiotus* sp. and *M. shonaicus*. A testicular spermatozoon of *Paramacrobiotus* sp. (*A*, *A′*) and *M. shonaicus* (*B*) with SEM. (*A′*) is an expanded image of (*A*). Scale bars indicate μm. a: acrosome, m: mid-piece, n: nucleus, t: tail.

Materials 2). Measurements of spermatozoa and eggs of *Paramacrobiotus* sp. and *M. shonaicus* were compared with a Multivariate Analysis of Variance (MANOVA) with default setting (Pillai–Bartlett statistic) of the 'manova' function in R (R Core Team, 2016).

Scanning electron microscope (SEM) images

The testicular spermatozoa of at least 10 males were collected from each species and observed. From each species, 15 females that laid eggs were selected for observations of the spermathecal spermatozoa and oocytes. The protocol for preparation and observation with an SEM was that described in Rebecchi and Guidi (1991).

At least 30 eggs of each species were collected, with at least 15 from eggs 5 min after being laid and at least 15 over 1 day after being laid. Eggs were dehydrated in 100% ethanol for 3 h and then placed in tertiary-butyl alcohol, soaked overnight, and lyophilized using a JFD-320 device (JEOL, Japan).

All samples were transferred onto aluminium stabs, sputter coated with gold, and observed using an SEM, JSM 6510 (JEOL). Person's chi-squared test with default settings (Yates's continuity correction) of 'chisq.test' function of R (R Core Team, 2016) was performed to compare the numbers of eggs containing spermatozoa (row = number of oocytes/laid eggs, column = number of specimens with/without spermatozoa, in each species).

Results

Morphological comparison of testicular spermatozoa

Testicular spermatozoa from both species had a tail with a tuft, mid-piece, coiled nucleus and acrosome (Fig. 1). The mid-pieces

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Table 1. Spermatozoa length

	Paramacrobiotus sp. (µm)				*M. shonaicus* (µm)			
	min	max	mean	SD	min	max	mean	SD
Tail	13.0	29.4	19.3	4.2	9.4	24.2	15.8	3.3
Mid-piece	1.5	6.6	3.7	1.1	1.1	3.6	2.3	0.6
Nucleus	13.2	35.0	21.4	4.3	6.2	18.2	10.8	2.5
Acrosome	24.5	52.6	38.5	6.7	0.6	2.9	1.6	0.5

of *Paramacrobiotus* sp. and *M. shonaicus* were kidney-shaped and rod-shaped, respectively. Morphometrics of the measured spermatozoa are available in Tables 1 and S1, and the lengths of the spermatozoa were significantly different ($F > 21.0$, $P < 0.00001$ in all measured with MANOVA; Fig. 2A and Table S2) between *Paramacrobiotus* sp. and *M. shonaicus*. The largest difference between the species was the length of the acrosomes: 37.6 µm in *Paramacrobiotus* sp. and 1.5 µm in *M. shonaicus* (median values, $F > 1515.0$, $P < 0.00001$ with MANOVA; Table S2*A*).

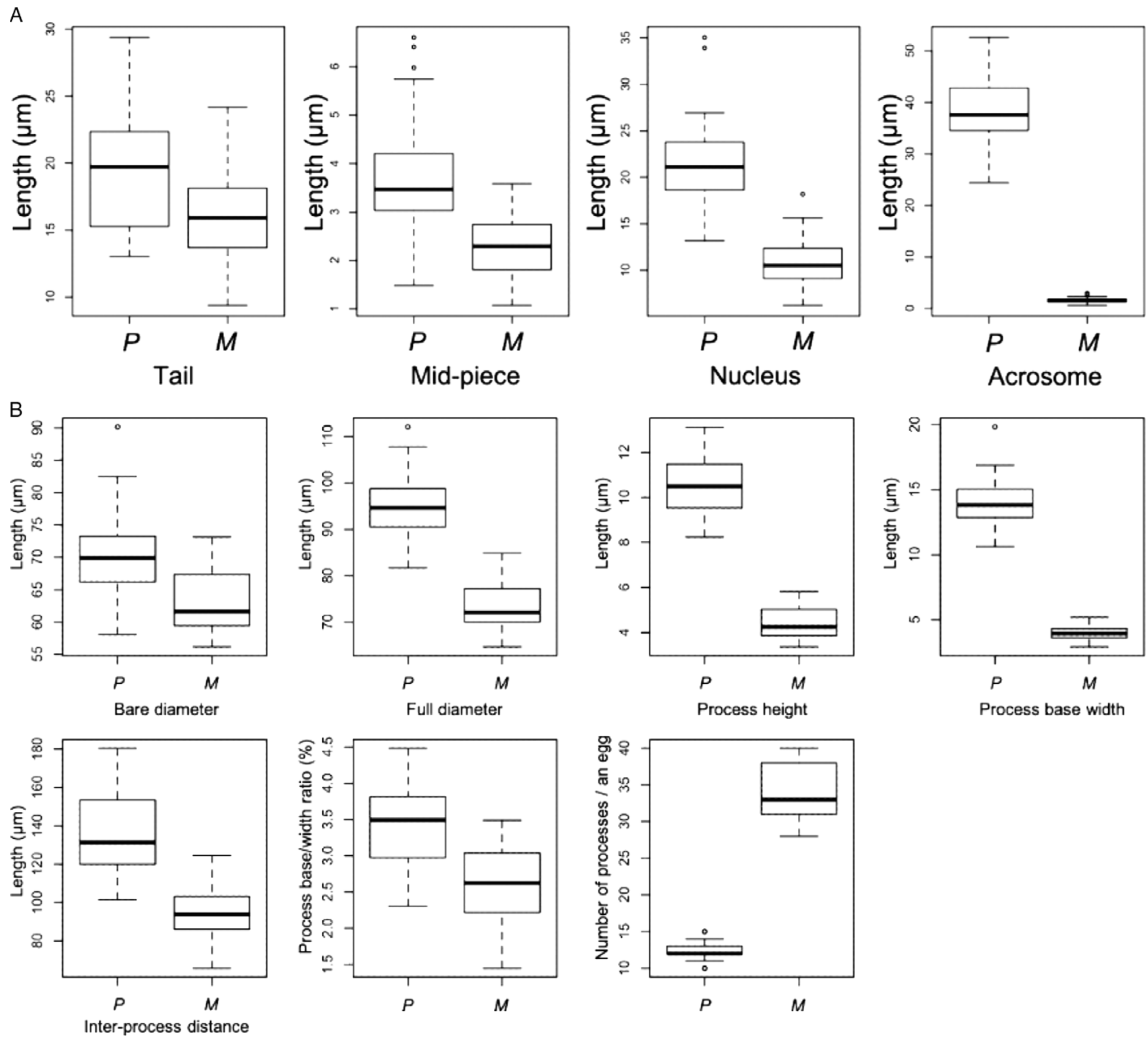

Figure 2. Length of four parts of a spermatozoon. Box plots of morphometrics of spermatozoa (*A*) and eggs (*B*). *P* and *M* indicate *Paramacrobiotus* sp. and *M. shonaicus*, respectively. MANOVA shows statistical difference in both spermatozoa and eggs.

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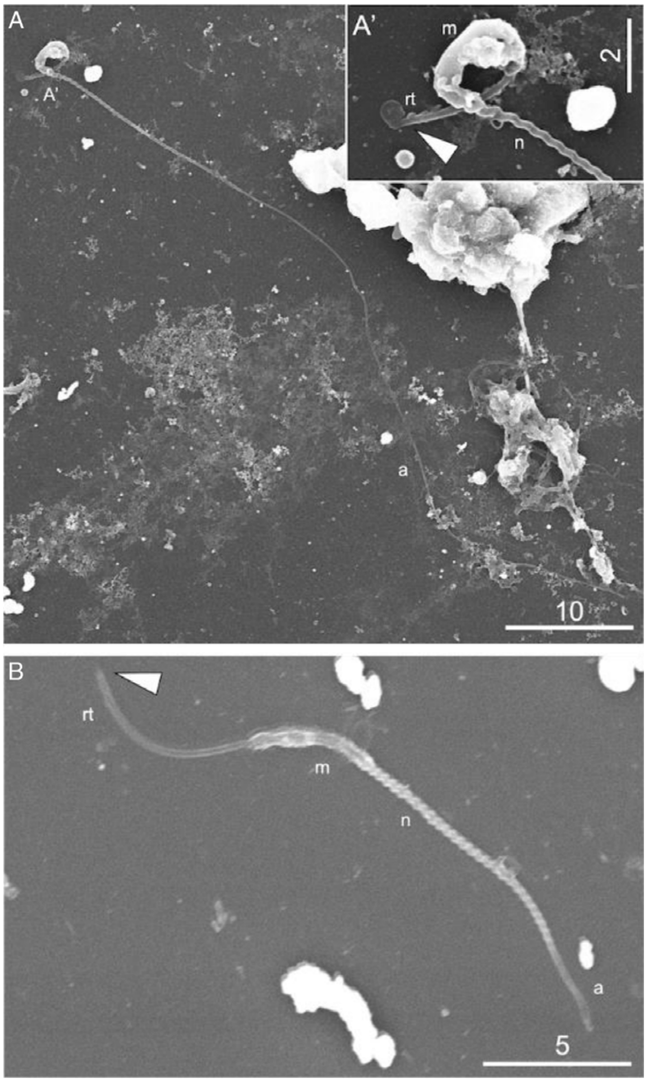

Figure 3. Spermathecal spermatozoa of *Paramacrobiotus* sp. and *M. shonaicus*. SEM image of a spermathecal spermatozoon of *Paramacrobiotus* sp. (*A*, *A′*) and *M. shonaicus* (*B*). (A′) is an expanded image of (*A*). Scale bars indicate µm. Arrow heads indicate reduced tails. a: acrosome, m: mid-piece, n: nucleus, rt: reduced tail.

Morphological changes in the spermathecal spermatozoa

All obtained spermathecal spermatozoa had reduced tails (Fig. 3). The length of the modified tail was 1.3–3.6 µm in *Paramacrobiotus* sp. (Fig. 3A, *A′*) and 3.0–4.6 µm in *M. shonaicus* (Fig. 3B and Table S3). The tails of spermathecal spermatozoa were significant shorter than the tails of testicular spermatozoa in both species ($F > 64.0$, $P < 0.00001$ with MANOVA; Table S2*B*). There were no differences in any other parts of the spermathecal spermatozoa.

SEM images of oocytes and laid eggs in a time series

In total, 22 and 20 oocytes from the *Paramacrobiotus* sp. and *M. shonaicus* laying females, respectively, were observed (Fig. 4). None of the oocytes with poorly-developed processes had spermatozoa in them.

The eggs collected 0–5 min after being laid were soft, elliptical, and flabby [Figs 5A and 6A; just-laid eggs (1 min), shown in Movie S1]. In addition, there were spermatozoa with reduced tails on the surface of the chorions on the areolae in both species (Figs 5B–D, 6B, C). The nuclei were coiled in the testicular and spermathecal spermatozoa (Figs 5B, C and 6B). Although the reduced tail, mid-piece and nucleus were clearly shown, the tip of the acrosome appeared buried in the chorion (Figs 5D and 6D). Spermatozoa with reduced tails were found on 44% (8/18) and 40% (6/15) of the observed eggs from *Paramacrobiotus* sp. and *M. shonaicus*, respectively. The number of eggs with spermatozoa was significantly different from the number of oocytes (chi-squared test, $P < 0.01$ in both species; Table S4).

Eggs collected over 1 day after being laid had hardened processes on their surface (Fig. 7A, B). In total, 10–15 conical processes were observed on the eggs from *Paramacrobiotus* sp. (Fig. 7A and Table S5), and there were many inverted-goblet shaped structures on the eggs from *M. shonaicus* (Fig. 7B). No spermatozoa were obtained from the surface. Statistical tests between the morphometrics of *Paramacrobiotus* sp. and *M. shonaicus* eggs showed significant differences in the measured structures ($F > 26.0$, $P < 0.00001$ using MANOVA; Fig. 2B and Table S2).

Discussion

Eutardigrades have no genitalia and therefore they oviposit and ejaculate from their cloacae (Bingemer *et al.*, 2016; Altiero *et al.*, 2018; Sugiura *et al.*, 2019). During dioecious mating behaviour, *I. dastychi* females lay eggs into the exuviae during moulting, and males ejaculate their spermatozoa during her moulting (Bingemer *et al.*, 2016). Males of the two species that we studied courted females and released their spermatozoa into the environment near the cloaca (Sugiura *et al.*, 2019). To reproduce, individuals have to recognize that the male or female is of the same species before mating. How males and females recognize each other is still unclear, however our observations of the two tardigrade species used in this study strongly supported a previous finding that females secrete a male-attracting pheromone (Sugiura *et al.*, 2019). In an experimental system with *I. dastychi*, male recognition for the pre-ovipositional female was indicated (Bartel and Hohberg, 2019).

Morphological diversity in spermatozoa has been confirmed in some species of Macrobiotidae (Rebecchi and Guidi, 1991; Guidi and Rebecchi, 1996; Rebecchi, 1997, 2001; Rebecchi *et al.*, 2011; Bertolani *et al.*, 2014). The genera *Paramacrobiotus* and *Diaforobiotus* have a longer acrosome, whereas the acrosome is shorter in the genera *Macrobiotus*, *Xerobiotus*, and *Mesobiotus*. Our observations of testicular spermatozoa in the two species was similar (Fig 1), indicating that spermatozoon shape is generally conserved in these genera. Based on our results, nucleus and acrosome lengths had the most influence in characterizing the spermatozoa of each species (Fig. 2). Therefore, nucleus and acrosome lengths might be considered important characters for defining species in Macrobiotidae. In addition, our results indicated that morphometrical variance existed not only in the eggs (Stec *et al.*, 2016; Sugiura *et al.*, 2020), but also the spermatozoa.

Ejaculated spermatozoa from these two species swim to reach the female cloaca, and are then stored in the spermatheca (Sugiura *et al.*, 2019). The tail is reduced in the female body, suggesting that a long tail obstructs storage and is no longer required for fertilization (Fig. 3). Rebecchi (1997) also came to this conclusion with *X. pseudohufelandi*. Moreover, the tails of three species of

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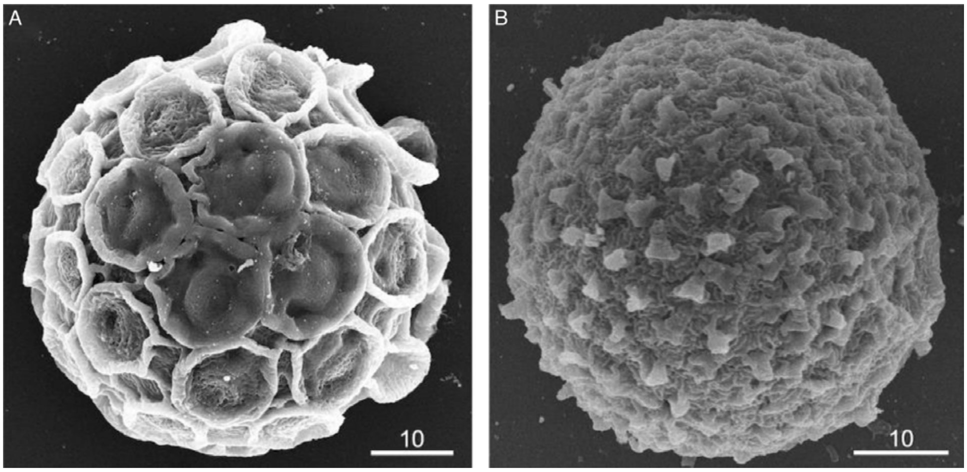

Figure 4. Oocytes of egg-laying females of *Paramacrobiotus* sp. and *M. shonaicus*. SEM images of oocytes from ovipositing females of *Paramacrobiotus* sp. (*A*) and *M. shonaicus* (*B*). Scale bars indicate μm.

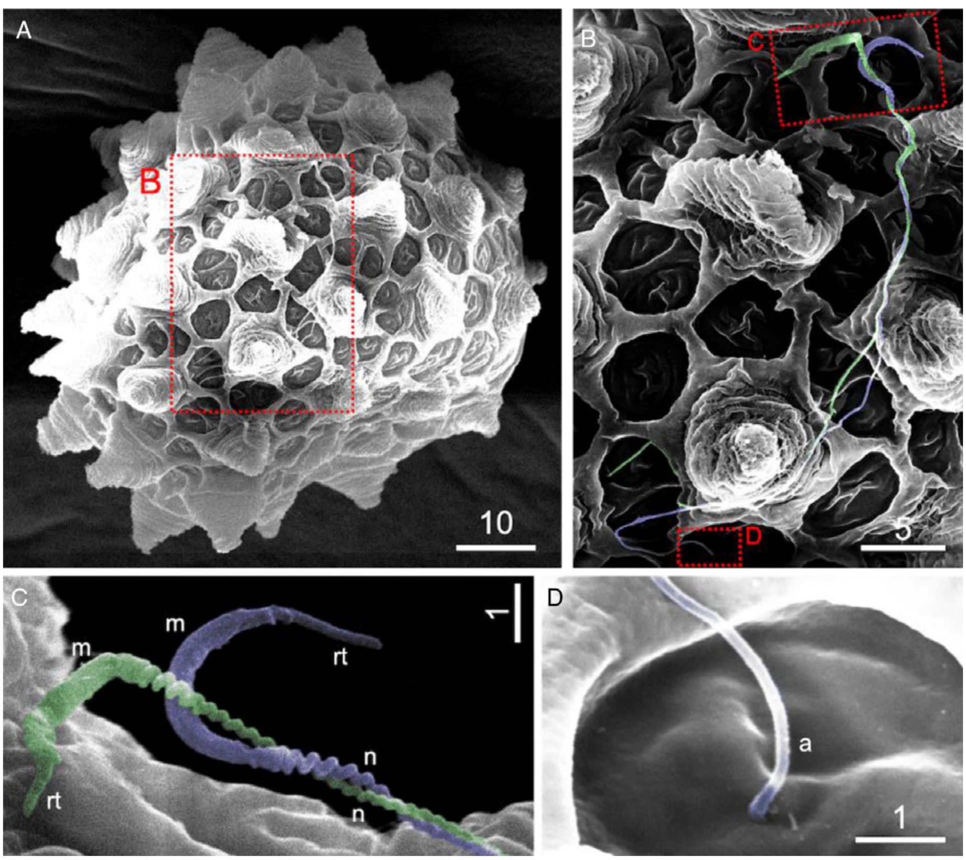

Figure 5. A laid egg with spermathecal spermatozoa from *Paramacrobiotus* sp. SEM image of a *Paramacrobiotus* sp. egg 5 min after being laid. Whole image (*A*), and expansion of red-dashed box of (*A*) and (*B*). (*C*, *D*) Expanded figures of red-dashed box of (*B*). a: acrosome, m: midpiece, n: nucleus, rt: reduced tail. Scale bars indicate μm.

Macrobiotidae – *X. pseudohufelandi*, *Paramacrobiotus* sp. and *M. shonaicus* – decreased in length in spermathecal spermatozoa, suggesting that the phenomenon is conserved at least among species of the family Macrobiotidae.

As reported by Guidetti *et al.* (2019), the spermatozoa were present on the chorion of the laid eggs. In addition, the spermatozoa on the chorion had reduced tails as in the spermathecal spermatozoa. Furthermore, although the penetration of the acrosome was not absolutely observed, the tip of the acrosome appeared buried the chorion, suggesting that the acrosome had penetrated.

In conclusion, we have reported measurements of gamete structure in two species of Macrobiotidae. There were morphometrical

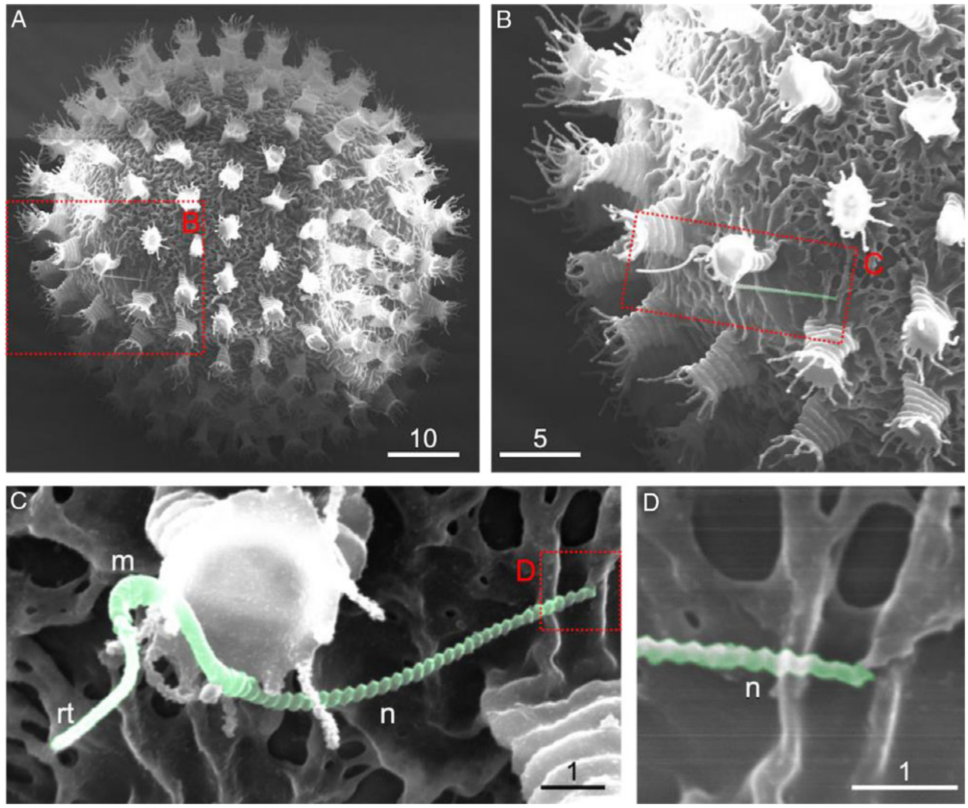

Figure 6. A laid egg with spermathecal spermatozoa of *M. shonaicus*. SEM image of an egg from *M. shonaicus* 5 min after being laid. Whole image (*A*), and expansion of red-dashed box of (*A*) and (*B*). (*C*, *D*): expanded figure of red-dashed box of (*B*) and (*C*), respectively. m: mid-piece, n: nucleus, rt: reduced tail. Scale bars indicate μm.

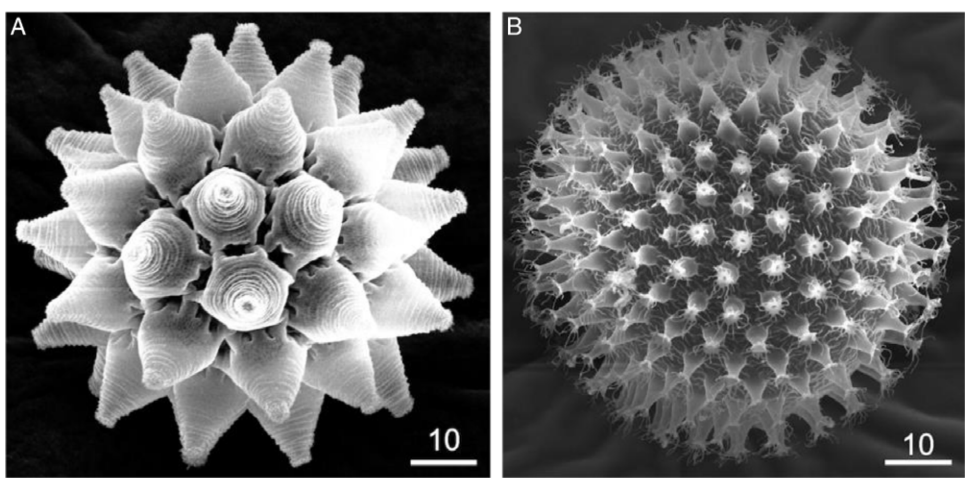

Figure 7. Eggs from *Paramacrobiotus* sp. and *M. shonaicus*. SEM images of eggs laid after more than one day, from *Paramacrobiotus* sp. (*A*) and *M. shonaicus* (*B*). Scale bars indicate μm.

variations in the spermatozoa between the species. In addition, our results suggest that the shortened-tail phenotype is conserved in three genera of Macrobiotidae. The first evidence in tardigrades of an acrosome penetrating the egg was also presented.

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Supplementary material. To view supplementary material for this article, please visit https://doi.org/10.1017/S0967199420000490

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Conflict of interest. None.

Ethical standards. Not applicable

Author contribution. KS performed all experiments and wrote the manuscript. MM improved the manuscript. Both authors designed this study.

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