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Life History of *Milnesium tardigradum* Doyère (Tardigrada) under a Rearing Environment

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ABSTRACT—A strain of carnivorous tardigrade, *Milnesium tardigradum*, was reared in water on agar plates at 25°C. The monogonont rotifer *Lecane inermis* was presented as a food source. This rearing system permitted detailed observation of tardigrade behaviour. Daily measurements of body length allowed the growth rate and moulting cycle of this species to be determined. The life history of *M. tardigradum* raised under these conditions included up to seven periods of moult. The first and second moults occurred at intervals of 4–5 days, and individuals reached reproductive maturity at the 3rd-instar stage; the first period of egg laying accompanied the third moult. The most rapidly developing animal in the study population laid eggs 12 days after hatching. The egg-laying intervals or moulting intervals of adult animals were around 6-10 days. The mean clutch size was 6.9 eggs. All tardigrades in this laboratory population were female and reproduced by parthenogenesis. The duration of the embryonic stage ranged from 5–16 days. The most long-lived female survived for 58 days after hatching, and laid a total of 41 eggs in 5 separate clutches. The entire life cycle of tardigrades reared under these conditions was recorded and photographed. A brief description of the embryonic development of *M. tardigradum* was also reported.

Key words: tardigrade, *Milnesium*, rearing, embryology, life history

INTRODUCTION

Terrestrial tardigrades show remarkable physiological adaptations that enable them to endure unfavourable environmental conditions. Tardigrades require moist environments to facilitate gaseous exchange and avoid desiccation. However, in drought conditions these animals can undergo active dehydration to form resistant structures called tuns. They are capable of surviving for prolonged periods in this state of desiccation-induced cryptobiosis, or anhydrobiosis. These physiological abilities were first observed during the eighteenth century and subsequent researches have revealed that tardigrades are also extraordinarily resistant to physical and chemical extremes (Wright, 2001). The phylogenetic relationships of tardigrades have attracted much interest, and several recent studies have considered their position within the Ecdysozoa (Garey *et al*., 1996; Giribet *et al*., 1996; Garey, 2001). However, there remains a notable absence of detailed information concerning the life history and embryology of these animals.

The difficulties involved in rearing tardigrades under experimental conditions have presented a significant obstacle to previous studies. Richters and Krumbach (1926) highlighted these problems as early as 1926. Although most of our current knowledge of tardigrade physiology and embryology is based upon Marcus' highly influential research (1927a, b, 1928, 1929), he was unable to follow these animals throughout their entire life cycle. Several reports have discussed methods for rearing tardigrades in culture, as reviewed by Altiero and Rebecchi (2001). However, detailed descriptions of their life history and embryology are still lacking.

Milnesium tardigradum Doyère, 1840 is a well-known cosmopolitan species of carnivorous tardigrade. Baumann (1964) described some aspects of the life history of this species on the basis of a 3-month study of a cultured population. However, the present study established a more desirable method for the rearing and observation of *M. tardigradum*. Daily measurements of body length elucidated the growth rate and moulting cycle of this species. A brief description of embryonic development was also made. This is the first report to present a comprehensive description of the life history of *M. tardigradum* reared in culture dishes.

MATERIALS AND METHODS

Milnesium tardigradum specimens were collected during April 2000 from moss, *Bryum argenteum*, growing on a wall of the laboratory building in which the author works (Fig. 1). Moss samples

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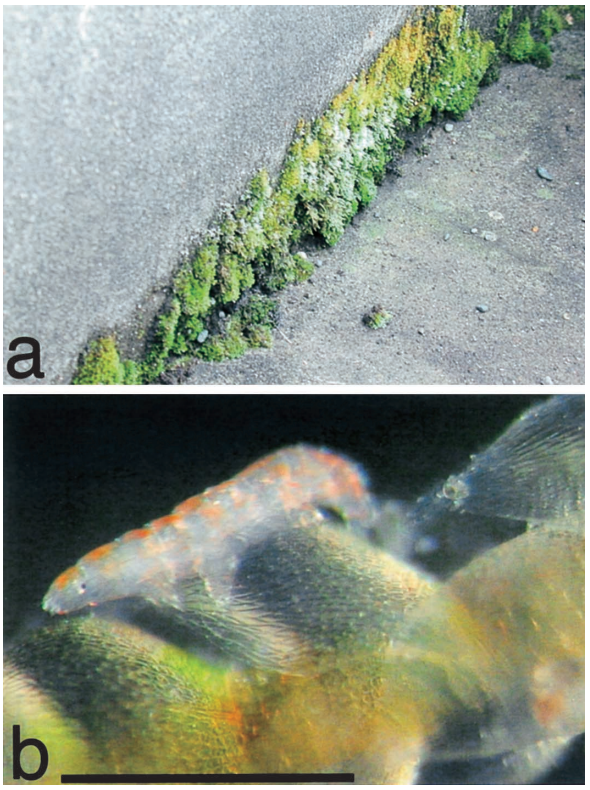

Fig. 1. Natural habitat of the tardigrade, *Milnesium tardigradum*. (a) Tardigrades were collected from moss growing on an external wall. (b) *M. tardigradum* walking on moss. Dorso-lateral view showing reddish-brown pigmentation. Anterior is to the left. Scale bar, 500 μm.

were soaked in water for several hours in order to release the tardigrades, which were recovered using a pipette under a stereo microscope. Tardigrades were reared in 3 or 6 cm plastic culture dishes. The bottom of each dish was coated with 2% agar (Difco) in KCM solution (7 mg KCl, 8 mg $CaCl_2$ and 8 mg $MgSO_4$-$7H_2O$ in 1,000 ml of water), and a layer of water (Milli-Q, Millipore) was added, to a height of 2-3 mm. The rotifer *Lecane inermis* (Bryce, 1892) was provided as prey. Rotifers were isolated from a xenic protozoan culture in the laboratory and were maintained with rice grain immersed in KCM solution. Identification of the rotifer species followed Segers (1995).

Live animals were photographed under bright field or differential interference contrast (DIC) microscopy using a Nikon Optiphoto microscope, or digital images were obtained using a camera (Coolpix 950, Nikon, Tokyo, Japan) set on a stereo microscope (Leica MZ Apo). Body length, from the tip of the head to the junction of the 4th pair of legs, was calculated using the public domain NIH Image program (http://rsb.info.nih.gov/nih-image/). In cases where individuals had been photographed whilst walking, or with bent bodies, body length was calculated using data from the most elongate image. As a result, these data may include some underestimated values.

RESULTS

Rearing conditions

Observations of moss samples in culture dishes revealed that tardigrades frequently encountered ciliates, nematodes and large bdelloid rotifers. Although their feeding behaviour could not be determined, tardigrades were observed consuming unidentified monogonont rotifers of around 100 μm in length. Therefore, the monogonont rotifer *L. inermis* (Fig. 2), isolated from a xenic culture of protozoa, was chosen as the prey species in this study. *L. inermis* has a soft lorica and is sufficiently small for even 1st-instar *Milnesium* larva to feed on (Fig. 3a). This rotifer species proved to be an effective diet for rearing *M. tardigradum*. Subsequent observations of the original moss samples revealed tardigrades consuming small bdelloid rotifers, suggesting that they were a natural prey species.

It was necessary to coat the bottom of each culture dish with agar, since tardigrades found it difficult to move across plastic surfaces and their mouthparts often adhered to the bottom of the dishes. In some experiments, the sides of the dishes were also coated with agar to prevent animals becoming trapped in the gap between the bottom layer of agar and the wall, and being asphyxiated. However, this allowed the tardigrades to climb above the water level, where they often dried up. Gaps in the agar could be avoided by ensuring that water was added to freshly prepared agar plates.

The rearing method established in this study is summarized as follows: 1) A thin agar plate was prepared in a 3-cm dish and water was added. 2) Tardigrades were introduced into the dish. 3) Rotifers in KCM solution were added as a food source. In addition, the water was changed each time the animals were fed, and they were checked daily. Although tardigrades could be maintained if fed once every 3 days, restricted feeding decreased the number of eggs per clutch.

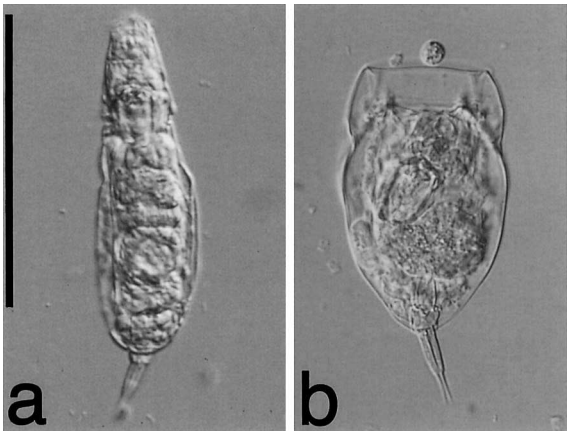

Fig. 2. The rotifer, *Lecane inermis*, presented as a food source for tardigrades during this study. Elongate (a) and contracted (b) form. Scale bar, 100 μm.

Feeding

Milnesium larvae were able to feed on rotifers from the 1st-instar stage, just after hatching, although they could not swallow entire prey until they had developed into 3rd instars. It frequently took over 15 min for larvae to absorb the con-

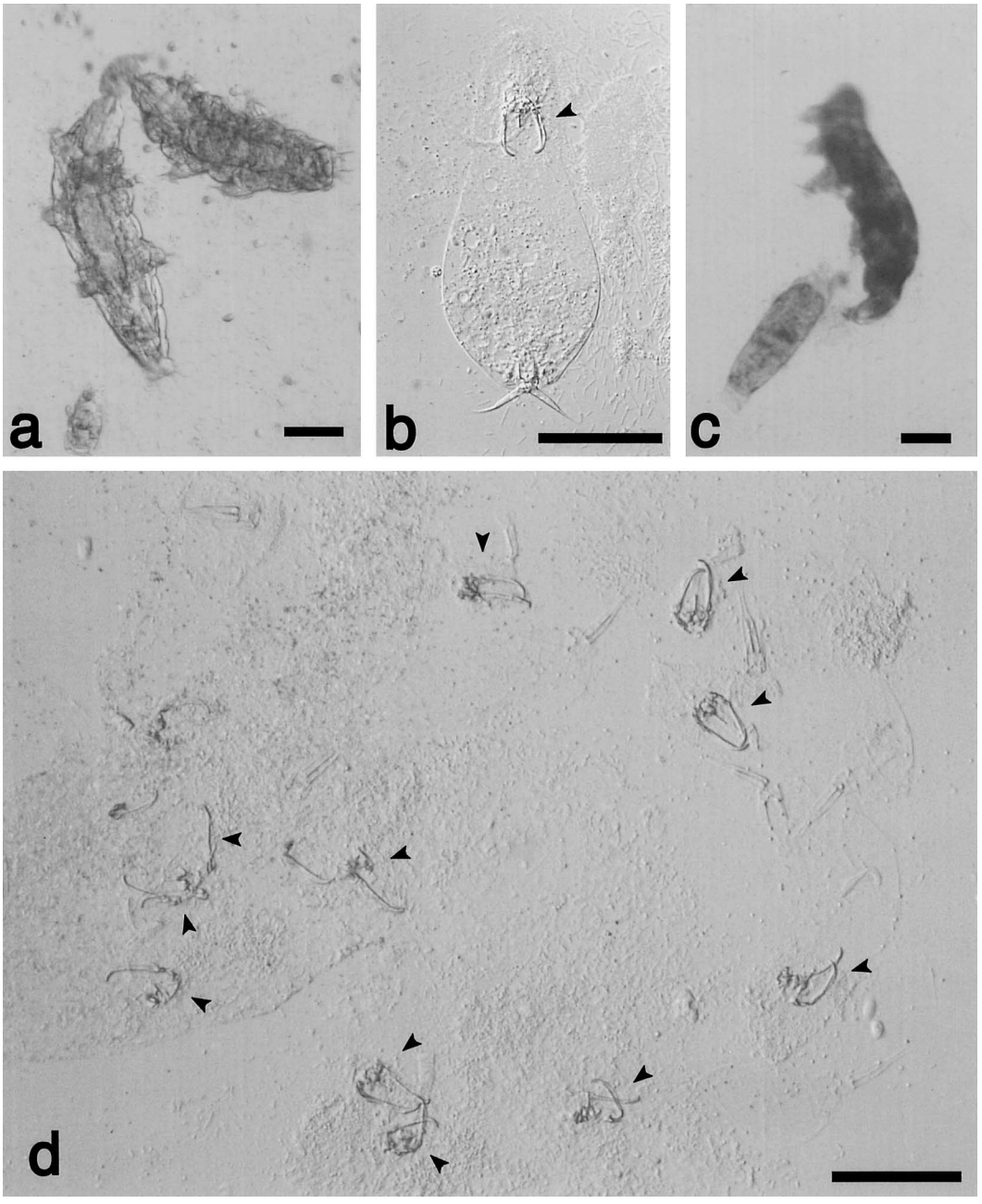

Fig. 3. Feeding of *Milnesium tardigradum*. (a) Two 1st-instar larvae eating a rotifer; scale bar, 100 μm. (b) Remains of a rotifer consumed by a 1st-instar larva; one trophi (arrowhead) remains in the lorica; scale bar, 50 μm. (c) A 4th-instar animal immediately after excretion; scale bar, 100 μm. (d) Contents of the faeces shown in c; eleven trophi (arrowheads) are present; scale bar, 50 μm.

tents of a rotifer through the lorica. Fig. 3b shows the remains of a rotifer that had been fed upon by a 1st-instar larva, leaving only the lorica, toes, and trophi.

Adult tardigrades were able to swallow rotifers whole. They actively searched for prey and abruptly attacked and swallowed rotifers as soon as they were within reach of their mouthparts. One adult animal consumed 13 rotifers successively over a period of 17 min; this individual then ceased feeding, although further prey was still available. Fig. 3c shows the faeces of a 4th-instar animal, containing the trophi of 11 separate rotifers (Fig. 3d).

Moulting and egg laying

The moulting process began with the 'simplex' stage, where tardigrades ejected the pharyngeal apparatus from the buccal tube to the oesophagus (Fig. 4) and the mouth opening was sealed. Individuals at this stage sought a safe place in which to settle, such as a crack in the agar. Ecdyses usually occurred on the following day.

All of the animals in this laboratory population were female and reproduction occurred by parthenogenesis. The tardigrades had translucent bodies, which permitted direct observation of the developing ovaries (Fig. 5). Egg laying

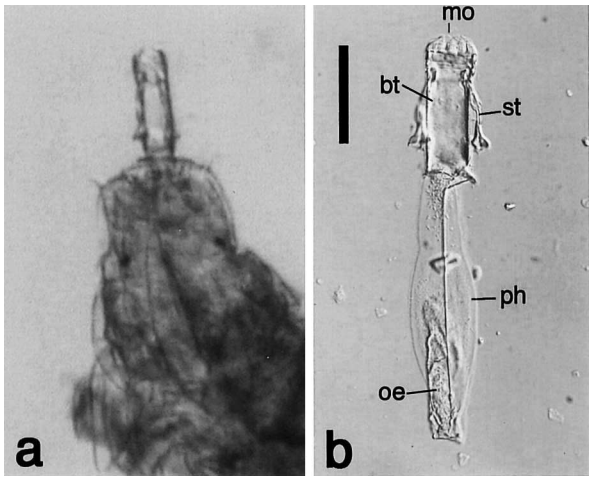

Fig. 4. Ejection of the pharyngeal apparatus. (a) A tardigrade ejecting its buccal tube. (b) A discarded pharyngeal apparatus; bt, buccal tube; st, stylet; mo, mouth opening; ph, pharynx; the oesophagus (oe) has been turned unnaturally upon the pharynx. Scale bar, 50 μm.

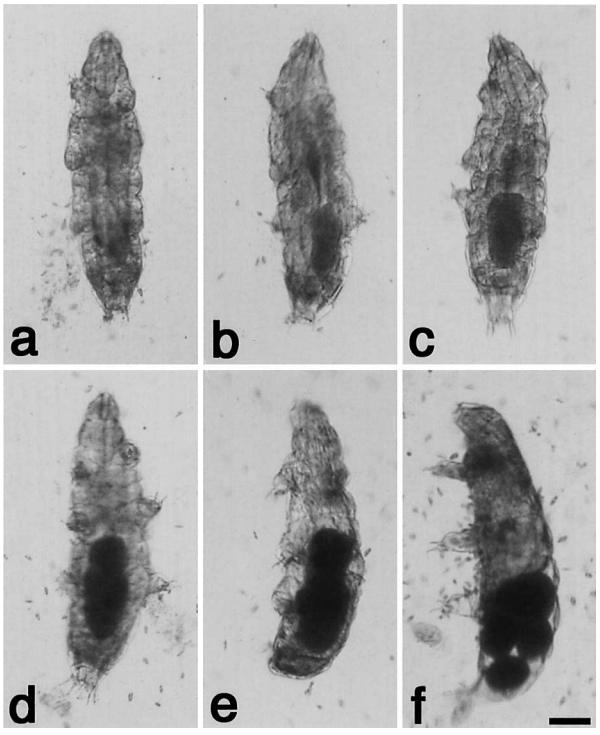

Fig. 5. Developing ovary of a 4th-instar animal at day 19 (a), day 20 (b), day 22 (c), day 24 (d), day 25 showing simplex (e), and day 26 (f). Anterior is to the top. Scale bar, 100 μm.

accompanied the moulting process and the eggs were laid in the space between the old and new cuticles. It was necessary for females to lift their tails in order to lay the eggs (Fig. 6), which underwent extreme constriction as they passed through the opening of the cloaca (Figs. 6b–d). The number of eggs in a clutch varied according to the condition of the mother and ranged from 1–12 eggs/clutch. The total duration of the egg-laying period (from the emergence of the first to the last egg) was less than 2 min in all cases. Females remained within the old cuticle, along with the developing embryos, for several hours after egg laying (Fig. 7). Occasionally, ecdysis did not occur and the mother died inside the old cuticle.

Moulting cycle and life span in culture dishes

Sixteen animals, from clutches containing 3, 6, and 7 eggs respectively, were observed daily from hatching until death to ascertain the details of their life history. Individuals within these broods were reared under the conditions described until they laid their first clutch of eggs; subsequently, each 4th-instar animal was removed and reared separately with daily feeds. Fig. 8 and Table 1 show the integrated results of these observations.

The first and second moult occurred at intervals of 4–5 days. Individuals reached maturity and were able to propagate after their second moult. All subsequent moults were accompanied by egg laying. Two of the 16 study animals died without reproducing. The remaining 14 individuals produced a total of 343 eggs in 50 separate clutches, with a mean clutch size of 6.9 eggs (Table 1 and Fig. 8). The most rapidly developing individual laid its first clutch 12 days after hatching (# 8 in Table 1). The average interval between clutches was 8.1 days (SD=2.2). These data varied widely between individual animals (Table 1), probably as a result of differences in nutritional condition.

The individual with the longest life span in this experiment (#16 in Table 1) remained alive for 58 days after hatching and laid a total of 41 eggs in 5 separate clutches. Towards the end of the experiment, this animal climbed out of the water and entered a state of anhydrobiosis on three separate occasions (day 55, 57 and 58). This individual was rehydrated twice, but observation was terminated when it formed a tun for the third time (day 58).

Embryonic development

The duration of the embryonic stage was determined for 127 of the 343 eggs produced during this study. Despite the constant temperature, the length of the developmental period varied widely between individuals, ranging from 5–16 days (Fig. 9). The hatchability was 77.2% (98/127 eggs). Those eggs that failed to develop appeared paler than those that developed normally when examined under transmitted light shortly after they had been laid.

Fig. 10 shows an example of the embryonic development of *M. tardigradum*. A clutch of 8 eggs was removed from the cuticle of one female after the first cleavage, which occurred 4 hr 30 min after egg laying (4:30 hr), and observed in detail. The first cleavage occurred vertically in respect to the long axis, and divided the embryo into two approximately equal blastomeres. The second cleavage (5:20–5:35 hr) was asynchronous, producing the 3-cell stage, and subsequent divisions also occurred asynchronously. Although it was difficult to observe the processes due to poor transparency of the embryo, the morula stage

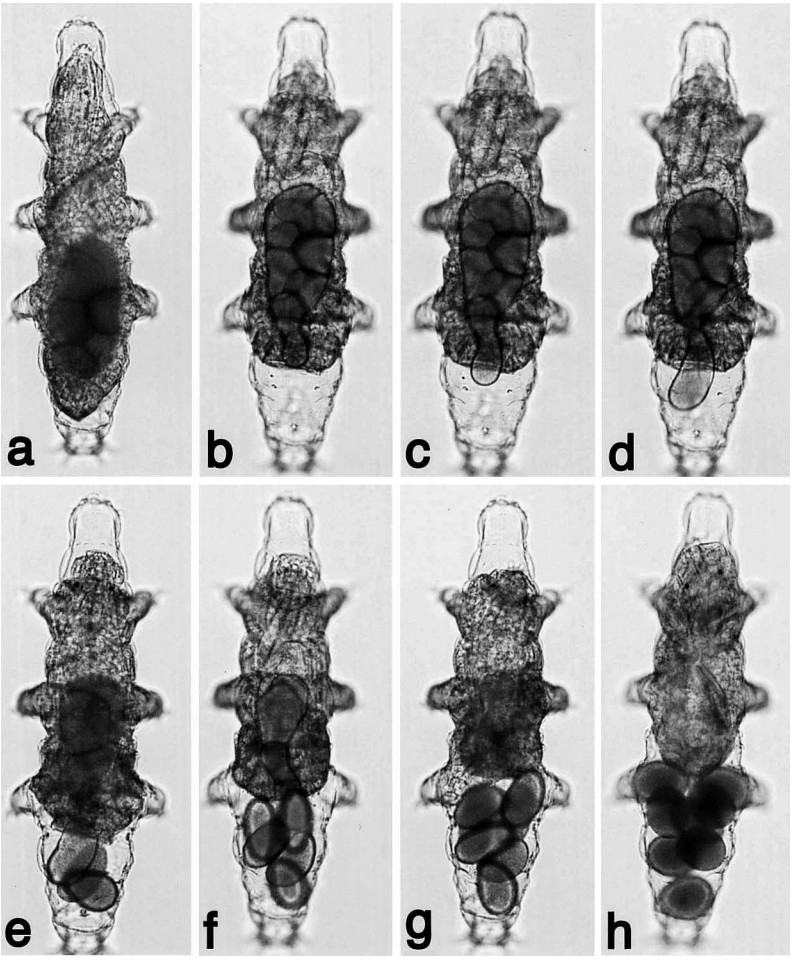

Fig. 6. Egg laying of *Milnesium tardigradum*. Anterior is to the top. A female, lifting her tail (a–b) to allow the first egg to emerge (b–d). Eight eggs laid in succession into the space between the old and new cuticles (e–h). The time interval between b and h is less than two min.

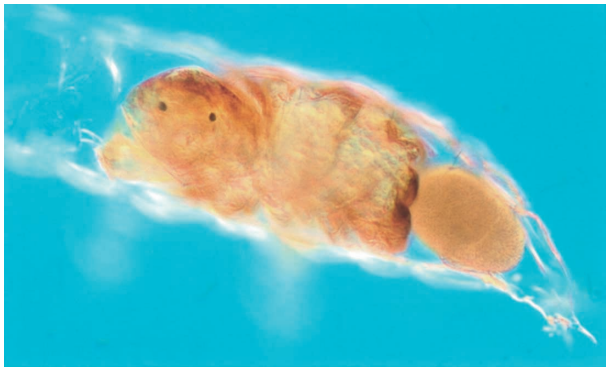

Fig. 7. Female tardigrade along with a developing embryo. Anterior is to the left. This animal laid only one egg. The embryo appears to be at the 4- or 5-cell stage. Females were able to move within the old cuticle before ecdysis, and this individual has turned to face the camera.

(8:25 hr) and the blastula stage (15:40 hr) were recognised. The outer layer of the embryo became slightly translucent and signs of morphogenetic movement were observed at around 41 hr. The photograph taken at 43:30 hr clearly shows the ventro-transversal cleft. The transparency of the embryo gradually increased with time and occasional movements of the embryo were observed at 96 hr. This movement became more active at 113 hr, by which stage the mouthparts were visible. The last two photographs (120 hr and 135 hr) show obvious mouthparts in the translucent embryo.

Shortly before hatching, the stylet of the fully developed embryo appeared to pierce the eggshell. Hatching occurred abruptly, after which the newborn larvae rapidly escaped from the exuvium (Fig. 11). Larvae eventually hatched from 7 out of 8 eggs in this clutch, 6 days after they were laid.

DISCUSSION

Baumann's studies (1964) and Schmidt's film (1971) are useful references concerning the biology of *M. tardigradum*. However, these works do not provide sufficient information to understand the life history of this species as they lack detailed descriptions of moulting, life span, and embryology. In one experiment, Baumann (1964) maintained a culture of *M. tardigradum* for up to 3 months, but was not able to distinguish individual animals in his study population.

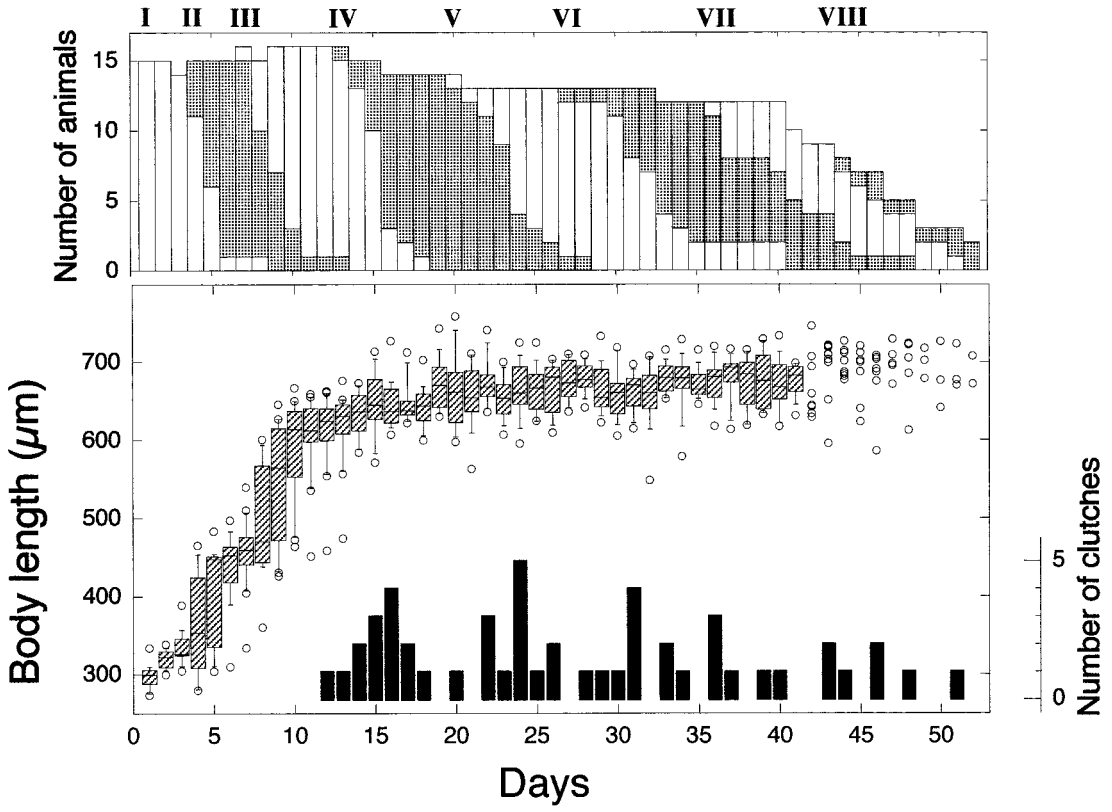

Fig. 8. Body length change (lower panel) and the number of individuals classified by instar (upper panel) in the culture dish at 25°C. Instars are indicated by Roman numerals. Missing data was a result of the difficulty of finding tiny larvae (up to day 8), missing animals (day 14), and the death of individuals (from day 16 onwards). The boxes in the lower panel (up to day 41) indicate the range between the 25th and 75th percentiles; the line in each box marks the median; capped bars indicate the 10th and 90th percentile points; and outlying points are shown with small circles. After the 3rd instar, each moult involved egg laying (histogram in the lower panel).

Table 1. Egg laying and life span of *Milnesium tardigradum*

#	Number of Clutches	Time (days) to each egg laying from hatching	Clutch size (eggs/clutch)	Number of total eggs	Life span (days)	Last active instar
1	–	–	–	–	14*	3
2	0	–	–	0	15	3
3	1	13	5	5	21	4
4	2	14, 22	3, 7	10	33	5
5	2	18, 28	6, 6	12	41	5
6	3	16, 24, 33	6, 8, 7	21	42	6
7	3	15, 25, 40	7, 6, 3	16	43	6
8	3	12, 20, 26	5, 7, 8	20	45	6
9	3	14, 22, 34	5, 8, 4	17	49	6
10	4	16, 24, 31, 43†	8, 7, 10, 3	28	43	6
11	4	17, 24, 31, 36	7, 10, 10, 11	38	52	7
12	5	16, 23, 30, 36, 46†	5, 8, 10, 10	37	46	7
13	5	15, 22, 31, 37, 48†	4, 6, 6, 9	28	48	7
14	5	17, 24, 29, 36, 44	5, 8, 11, 6, 6	36	47	8
15	5	16, 24, 31, 43, 51	7, 9, 6, 4, 8	34	57	8
16	5	15, 26, 33, 39, 46	9, 6, 7, 11, 8	41	58<	8

Data of sixteen animals are sorted by the number of clutches and their longevity. * Missing before day 14 † Died without ecdysis

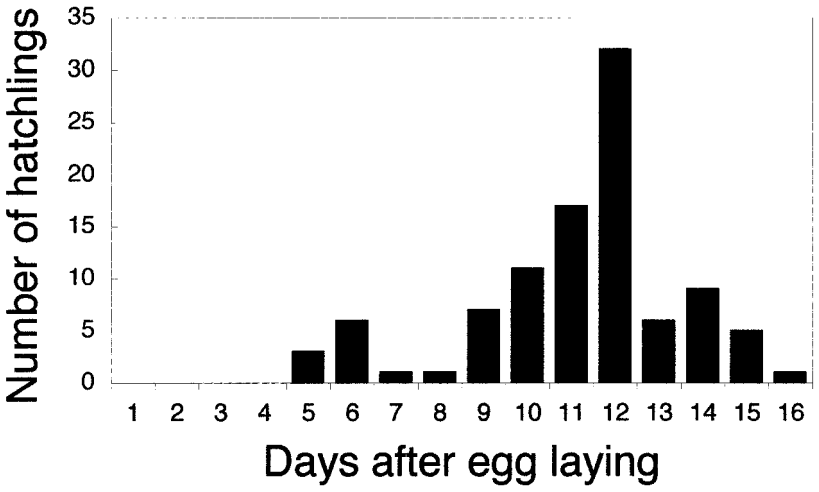

Fig. 9. The time interval (days) between egg laying and hatching (at 25°C).

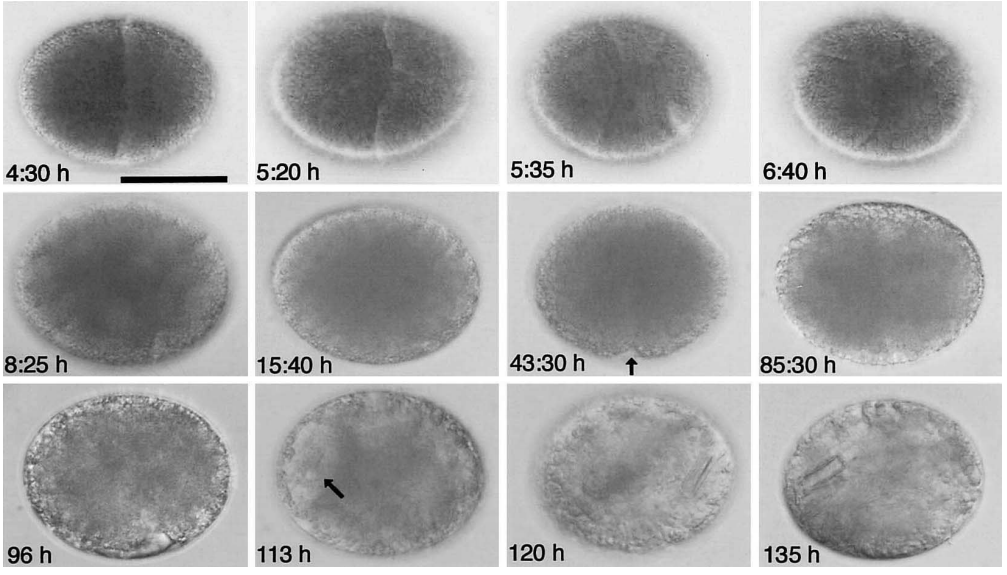

Fig. 10. An example of embryonic development of *Milnesium tardigradum*. Time since egg laying is shown in the lower left corner (N.B. the duration of embryonic development showed wide individual variation). The first cleavage produced the 2-cell stage (4:30 hr). Subsequent asynchronous divisions produced the 3-cell (5:20 hr), 4-cell (5:35 hr), and 5-cell stages (6:40 hr). Cleavage produced the morula (8:25 hr) and the blastula (15:40 hr). Morphogenetic movement produced the ventro-transversal cleft (43:30 hr). A ventral view (85:30 hr). Increasing transparency and occasional rotatory movement (96 hr). The mouthparts are partially visible at 113 hr (arrowhead) and clearly visible by 120 hr. The embryo shortly before hatching (135 hr). Scale bar, 50 μm.

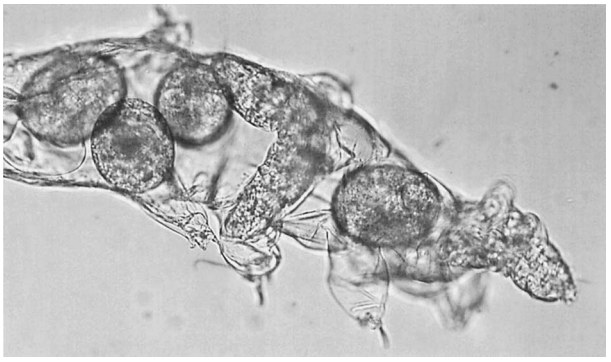

Fig. 11. Newly hatched larvae escaping from the exuvium. One larva is about to emerge, whilst a second searches for the exit hole.

In a separate experiment, Baumann described aspects of the egg-laying behaviour of an isolated animal. This individual produced a clutch 40 days after hatching and the next maturation of the ovary was observed at 53 days. However, no additional observations of this individual were recorded and Baumann did not describe its complete life cycle. Although body length frequency has been used to determine the number of instars in some tardigrade species (Higgins, 1959; Sayre, 1969), this method was not applicable to *M. tardigradum* (Baumann, 1964; the present study). Previous researchers have been unable to directly observe tardigrades during moulting because rearing methods involved complicated environments including moss and algae (Marcus, 1927b; Baumann, 1964). However, the present study

established a rearing system that permitted direct observation of the behaviour of *M. tardigradum*. Individual females reared under the conditions described underwent up to 7 moults during their life cycle. The system also allowed data on the embryonic development and post-embryonic growth of this species to be collected. This is the first published report to include a comprehensive description of the life history of *M. tardigradum* raised in culture dishes.

The rearing conditions described here differed from the natural habitat of *M. tardigradum* in a number of ways. Active individuals were obliged to move across the broad, flat agar plates to search for food. However, during the simplex stage individuals favoured interstices or holes in the agar formed from scratches on the surface created during water changes; the exuvia and eggs were often found in such crevices. This reflected the natural behaviour of these animals in their original moss cushion environment. The constant wet conditions maintained in the culture were also highly artificial, and may not have been optimal for rearing *M. tardigradum*; several authors have reported that this species favoured mosses that frequently dried out (Ramazzotti and Maucci, 1983; Wright, 1991). In cases where the sidewalls of the culture dishes were coated with agar, tardigrades repeatedly crawled out of the water and entered a state of anhydrobiosis; missing animals were often found above the water level. This behaviour raised the question of whether the animals required periods of desiccation. Previously, Rahm (1927) reported a negative correlation between constant hydration and life span in tardigrades. Therefore, the possibility remains that anhydrobiosis functions as a positive interval of rest and refreshment as well as providing protection against adverse environmental conditions. Crowe and Higgins (1967) investigated the revival time for anhydrobiotic individuals of *Macrobiotus areolatus*, and suggested that well-fed tardigrades revived more rapidly than starved ones. Entering a state of anhydrobiosis when in a favourable nutritional condition may be a strategy for prolonging longevity. However, the detailed effects of anhydrobiosis on life span have not yet been determined.

Although it has been assumed that tardigrades have very long life spans, little information is available concerning their longevity. The well-known legend that a tardigrade revived after a period of anhydrobiosis lasting 120 years is an exaggeration of the original report (Franceschi, 1948; Jönsson and Bertolani, 2001). Nevertheless, it is possible to estimate tardigrade life span by assuming repetitive cycles of activity and cryptobiosis. For example, if tardigrades have at least 50 active days at 25°C, and the loss of energy during anhydrobiosis is negligible, the total life span of an animal that is active for only 1-day per week could extend to almost 1 year. In practice, life span may exceed this estimation at lower temperatures when development may proceed more slowly, or if anhydrobiosis has a positive effect on longevity. In the case of the triploid strain of *Macrobiotus richtersi* with an active life span of up to 330 days at 14°C (Altiero and Rebecchi, 2001), the total longevity of an animal that is active for only 1-day per week could exceed 6 years. Animals that undergo periods of anhydrobiosis during their life cycle and have a thelytokous mode of reproduction may evolve slowly. The extreme similarities between *Milnesium swolenskyi* found in amber from the Upper Cretaceous (90 million years old) and modern *M. tardigradum* (Bertolani and Grimaldi, 2000) suggests that anhydrobiosis may have a freezing effect on the molecular clocks of these tardigrades.

The large size of *M. tardigradum* eggs was an advantage when studying their embryology, although Marcus (1929) noted that it was difficult to observe cleavage in this species because of the bulky yolk granules in the egg. The present study confirmed that the egg was not transparent. However, it was possible (with difficulty) to distinguish the cleavage furrow. It may also be possible to obtain full details of the cleavage pattern of *M. tardigradum* by taking internal images of eggs. The duration of embryonic development in the present study varied considerably between individuals; the most delayed embryo took 16 days to develop in contrast to the shortest development period of just 5 days. Cleavage time may also have varied between individuals. Variation was not caused by genetic factors, since thelytokous animals are genetically identical. The stage at which developmental retardation occurred, and the factors responsible, are not known, although anoxia may influence embryonic development (Altiero and Rebecchi, 2001).

Finally, it should be noted that a strain originating from the most long-lived animal in this study (#16 of Table 1) has been maintained since autumn 2000, with good hatchability (about 90% in summer 2002).

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