Effect of female aging on the morphology and hatchability of resting eggs in the rotifer *Brachionus plicatilis* Müller

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This paper has not been submitted elsewhere in identical or similar form, not will it be during the first three months after its submission to Hydrobiologia.

Abstract

This study examined the morphology and hatchability of *Brachionus plicatilis* resting eggs as a function of the aging of maternal fertilized mictic females. One-hundred twenty fertilized *B. plicatilis* (Australian strain) were individually cultured and monitored daily until death. All cultures were maintained at 25°C, 11 ppt, and fed the micro-algae *Tetraselmis tetrathele*. Resting eggs produced by the females were investigated using two parameters: egg morphology and hatching rate. Under these culture conditions, females normally produce 1 to 6 (mean $\pm$ SD=2.7 $\pm$ 1.2) resting eggs during their lifetime. However, the number of resting eggs with abnormal morphology increased as a function of maternal age. Among resting eggs with normal morphology (n=225), 82.2% were produced during the first and second spawning, and had hatching rates of more than 60%, while the hatching rates were below 30% in resting eggs with a spawning order of >2. Thus, the quality of *B. plicatilis* resting eggs was negatively correlated with maternal age.

Introduction

The monogonont rotifer *Brachionus plicatilis* has a heterogonic life cycle comprising both asexual (amictic) and sexual (mictic) reproductive cycles. In asexual reproduction, amictic females parthenogenetically produce female offspring without male intervention, whereas oocytes of mictic females undergo full meiosis to produce haploid males or, if fertilized, diploid resting eggs, which are encased within a resistant cyst wall in sexual reproduction (Gilbert, 1974; Hagiwara & Hino, 1989; Wallace et al., 2006). Resting eggs are important to the evolutionary success of rotifers because of their dormancy capabilities, ease of dispersal and contribution to genetic variation through recombination (Pourriot & Snell, 1983).

The quality of resting eggs decreases in sediment as morphological characteristics deteriorate, and hatchability declines with sediment depth because of egg aging. However, low quality resting eggs are also observed in upper (younger) sediments (García-Roger et al., 2005). This means that the quality of resting eggs is affected not only by egg age, but unknown factors.

Several studies have shown that female rotifers undergo physiological changes with age (Clément & Pourriot, 1980; King, 1969; King & Miracle, 1980; Luciani et al, 1983; Pagani et al., 1993; Ricci et al., 1999). Jennings & Lynch (1928) published the first major study of aging effects on the survivorship and variation in parthenogenetic egg production in the monogonont rotifer *Proales sordida*. Thus, the aging of fertilized mictic females can be hypothesized to affect the morphology and hatchability of resting eggs. In the present study, we defined the quality of resting eggs based on morphological characteristics as well as hatchability, and examined the relationship between the aging of fertilized mictic females and the quality of resting eggs.

Materials and Methods

We used the Australian strain *Brachionus plicatilis* Müller, which has exhibited high sexual reproduction (mixis) induction and fertilization rates in our laboratory. To optimize the mixis, we cultured the rotifers at $25.0 \pm 0.1^\circ\text{C}$ and 11 ppt in total darkness (Hagiwara et al., 1988), and added the same amount of food daily (at a density of 0.24×10^6 *Tetraselmis tetrathele* cells/ml). Rotifer culture water (11 ppt) was prepared by diluting natural seawater with mili-Q water (Millipore $0.22\ \mu\text{m}$) followed by GF/C filtration and sterilization (121°C , 15 min.). This procedure allowed the stock culture to grow exponentially and reach the high population density required to induce mixis. When the stock culture showed active mixis induction, 120 amictic and 30 unfertilized mictic females were pipetted out and transferred separately to two petri dishes containing 10 ml stock culture conditioned media. A total of 296 female and 171 male neonates (<3 h) were transferred to another petri dish for fertilization. After 24 h, 120 fertilized mictic females with yellowish maturing oocytes in the coelom were individually transferred to 24-well polystyrene microplates containing 2 ml diluted seawater and food, and were then cultured under the same condition as the stock culture. The rotifers were transferred to new culture media containing fresh *T. tetrathele*. The food suspension culture medium was renewed daily in order to avoid contamination of protozoa and fungi, and the production and morphological characteristics of resting eggs were observed under stereomicroscope at that time (Olympus, SZX-ILLD2-100). Normal resting eggs (Fig. 1a) were distinguished by a regular oval shape with extra-embryonic space and a light brown embryo (Wurdak et al., 1978), while abnormal resting eggs (Fig. 1b) were an irregular shape, less compact and had a dark brown embryo. After judgment of morphological characteristics, the resting eggs were individually transferred to 96-well polystyrene microplates containing 200 μl 11 ppt seawater and immediately incubated at $25 \pm 0.5^\circ\text{C}$ under 3000 lux (24L:0D). Hatching

of the resting eggs was monitored daily and the hatching rate calculated.

The morphological characteristics of the resting eggs were analyzed in relation to their spawning order, using the chi-square contingency test followed by Tukey's wholly significant difference test using R version 2.6 (Zar, 1999). The relationship between the spawning order of resting eggs and the hatchability against the total number (normal and abnormal) of produced resting eggs, and against the number of produced normal resting eggs was analyzed using the Spearman rank correlation test using Statview version 5.0.

Results

Morphological characteristics and spawning order of resting eggs

A total of 120 fertilized mictic females were used in this experiment. Their mean life span $\pm$ standard deviation was 13.3 ± 2.8 days (range 2–19 days) and fecundity was 2.6 ± 1.2 eggs (range 0–6 eggs). One-hundred sixteen fertilized mictic females produced more than one resting egg, and other 4 individuals did not produce resting eggs until they died. Once the maternal females started producing abnormal eggs, most stopped producing normal eggs; only 4 exceptions among 51 individuals that produced abnormal resting eggs.

A total of 225 normal resting eggs were produced, with 82.2% produced as first and second eggs (Fig. 2). All resting eggs that were produced first were normal (mean time to spawning, 3.6 ± 1.2 days, $n=116$). Abnormal resting eggs began to appear from second eggs (mean time to spawning, 5.1 ± 1.2 days, $n=99$), while the number of abnormal resting eggs was the highest (49.3%) at third egg (mean time to spawning, 7.3 ± 1.7 days, $n=65$). Production of resting eggs with abnormal morphologies

increased over a lifetime when the fecundity of the maternal females increased. Production of normal resting eggs reached 0% in the fifth (mean time to spawning, 9.0 ± 1.4 days, $n=7$) and sixth spawning order (mean time to spawning, 12.0 ± 0.0 days, $n=2$) (Fig. 2). Thus, spawning order was a significant factor ($p < 0.001$) in determining the morphology of the resting eggs.

Hatchability and spawning order of resting eggs

Only resting eggs that were determined normal morphology hatched. The highest hatching rate was found in first spawned eggs (73.5%) against the total number of resting eggs (normal and abnormal) produced. Thereafter, hatching rates declined significantly ($p < 0.05$) as a function of spawning order: 73.5, 61.4, 31.3, 12.5, 0 and 0%, respectively. Moreover, the hatchability against the number of produced normal resting eggs was also the highest at the first spawned resting egg group (73.5%), followed by 71.3, 61.8, 50.0, 0 and 0%, respectively (Fig. 3).

Quality of resting eggs and fecundity of maternal females

Only normal resting eggs were produced by rotifers whose fecundity was one egg, while no rotifers produced only abnormal resting egg (Fig. 4). Abnormal resting eggs were produced by rotifers spawned more than 2 eggs during their lifetime. High fecundity rotifers (≥ 3 eggs) had more than 58% possibility to produce abnormal resting eggs, and produced low hatchability resting eggs (less than 50%). We obtained only 2 individuals with a fecundity of 6 eggs, and these hatching rates were 83%.

Discussion

The resting eggs of rotifers such as *B. plicatilis* sink to the bottom and become buried in sediment, where they may remain for decades (Hagiwara et al., 1985; Kotani et al., 2001; García-Roger et al., 2005) and thus may be altered by the prevailing conditions. García-Roger et al. (2005) reported that rotifer resting eggs in sediment have different hatching success associated with morphological characteristics such as shell integrity, embryo size and color of the resting eggs. In their study, healthy-looking resting eggs were found in upper sediment layers, whereas a higher incidence of deteriorated resting eggs was observed as sediment depth increased. Thus, aging of resting eggs in sediment affects their quality (morphological characteristics and hatchability). However, because deteriorated resting eggs were also found in upper (younger) sediments, the quality of resting eggs might be associated with factors other than egg age, although these factors have not yet been clarified. Among newly formed resting eggs in laboratory, Hagiwara et al. (1985, 1989, 1995) and Hagiwara and Hino (1989, 1990) used only normal resting eggs for hatching. This was because abnormal resting eggs do not show hatchability, even though they were observed commonly.

In this study, aging effect on morphological characteristics and hatchability of resting eggs was investigated, and we found that the quality (morphology and hatchability) of resting eggs was negatively correlated with maternal age. First spawned resting eggs (mean time to spawning, 3.6 ± 1.2 days) were high quality, with a normal shape and high hatchability, and thereafter the quality rapidly decreased. At the fifth and sixth spawning, normal shaped resting eggs ceased to be produced (Fig. 2). Thus, the quality of resting eggs decreased with the age of maternal fertilized mictic females.

Hatchability was obtained in two ways: first by calculating the number of hatched eggs against the total number of resting eggs (normal and abnormal) and then against the total number of normal resting eggs (Fig. 3). Resting eggs produced by aged

females who experienced more spawning showed lower hatchability in both cases. Thus, the hatching of resting eggs without any sign of morphological alternation is affected by maternal age. These results confirm that the aging of maternal females leads to the deterioration of not only the morphology but the cytoplasmic quality of normal eggs. Therefore, it is probable that egg banks contain resting eggs that have deteriorated not only due to egg age but to the age of the maternal fertilized mictic females.

Higher hatching rates of resting eggs have been reported for natural systems. Hagiwara et al. (1985) observed 95 to 100% hatching success of resting eggs collected from an outdoor pond, and Hagiwara & Hino (1990) reported that a fertilized mictic female produced 1 to 8 resting eggs with a more than 90% hatching rate. However, we optimized the culture conditions for resting egg production and hatching, and the hatching rate remained at about 70%. Hagiwara & Hino (1990) reported that resting egg hatching rates increases from 20 to 90% when rotifers are fed *Tetraselmis* over several generations. Thus, nutritional and genetic mechanisms may be involved in the improvement of resting egg quality, and it is clear that female age does not always result in a deterioration of resting eggs.

In this study, the low fecundity (production of 1 egg) maternal females spawned only normal resting eggs with high hatchability, while the high fecundity (≥ 2 eggs) maternal females spawned normal resting eggs together with abnormal resting eggs, and the hatchability of those normal eggs decreased with the fecundity of maternal females. Thus, the fecundity of fertilized mictic females should be the other deteriorative factor of resting egg quality (Fig. 4).

The fecundity of fertilized mictic females in this study ranged from 0 to 6 with average of 2.6 ± 1.2 eggs. Most fertilized mictic females produce only two or three resting eggs, corresponding to the fact that males deliver about three sperms per copulation, and this means that fertilization is hard to occur more than twice per single

female (Snell & Childress, 1987; Snell et al., 2007). Thus, if fertilized mictic female rotifers have the capability to store sperms in the coelom for next spawning, then the aging of stored sperm should affect the quality of resting eggs.

This study indicates that the quality of resting eggs decreases as a function of the aging of the maternal female. However, we do not yet know the etiology of the abnormal morphological characteristics and poor hatching rates in rotifer resting eggs. Studies of potential factors, including sperm age and intrinsic properties of resting eggs produced by older females with reduced energy content, are needed to further this investigation.

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Figures

(a)

(b)

Figure 1. Morphological characteristics of resting eggs defined by shape and color. (a) Normal resting eggs which are a regular oval shape with extra-embryonic space and a light brown embryo. (b) Abnormal resting eggs which are an irregular shape, less compact and have a dark brown embryo.

Figure 2. Number of normal and abnormal resting eggs as a function of spawning order. Lower-case letters in the bar graph indicate significant differences ($a > b$, Tukey's wholly significant difference test, $p < 0.05$).

Figure 3. Hatchability of resting eggs against their spawning order. Bars with slant lines indicate hatching rate as a function of the total number of resting eggs (normal + abnormal); white bars indicate hatching rate as a function of the number of normal resting eggs (Spearman rank correlation test, $p < 0.05$).

Figure 4. The number of fertilized mictic females and hatching rate are plotted against the fecundity of fertilized mictic females. A line indicates the hatching rate of normal resting eggs, which were produced by maternal females with different fecundity.