Mate Recognition of the Rotifer *Brachionus plicatilis* Müller at Different Temperatures

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Recent studies on sexual isolation among euryhaline *Brachionus* species suggest that differences in the molecular structure of a mate recognition pheromone (MRP) are important in maintaining reproductive isolation. It has not been clarified whether environmental conditions can affect MRP structure and the responsiveness. The objective of this study was to examine the effect of temperature on mate recognition of the rotifer *B. plicatilis*. Three strains (Russian, German, and Tokyo) of *B. plicatilis* were cultured on *Nannochloropsis oculata* and a series of intra- and interstrain matings were made using female and male rotifers reared at three temperatures, 15, 20 and 25℃. Matings were conducted for nine combinations of treatments (3 strains x 3 temperatures). Temperature affected the attempted matings (frequency of circling behavior per male/female contact) differently among strains and sexes. Culture temperature of females affected the frequency of mating attempts. Male recognition of females occurred best at 20℃ for Russian strain. German strain males only recognized females when females were reared at less than 20℃. For Tokyo strain, however, rearing temperature of females did not affect mate recognition. MRP antibody binding did not correlate with the frequency of male mating attempts at each temperature.

Key Words : Rotifera, *Brachionus plicatilis*, temperature, mate recognition pheromone, mating attempts, copulation, antibody

Mating behavior of male monogonont rotifer is initiated after chemoreception of a signal on the body surface of conspecific females.[1-2] Males have chemosensory receptors on their corona[3] which recognize mate recognition pheromone (MRP), a surface glycoprotein on females.[4-6] Male display different frequencies of mating behavior with females of different geographical strains.[2,7-10] MRP is a surface glycoprotein with 29 kDa molecular weight,[11] whose protein and carbohydrate structure varies among rotifer strains, causing differences in mating frequencies.[10,12]

Kotani *et al.*[10] compared male mate recognition on females using three *B. plicatilis* and four *B. rotundiformis* strains. They observed a very low mating frequency in intrastrain crosses using a *B. plicatilis* strain from Germany. This raised the question of how the testing environment could affect male mating, perhaps by altering the MRP or its receptor. The objective of this study was to examine the effect of temperature on mating behavior of the rotifer *B. plicatilis*. We observed the mating behavior of the *B. plicatilis* strains cultured at different temperatures and examined the characteristics of the MRP at each temperature.

Materials and methods

Experimental strains were collected from different geographic regions. Three *B. plicatilis* strains were from Japan (Tokyo), Germany and Russia. Tokyo and German strains were referred to as J-TKU-II and WG-SCHL in the study of Fu *et al.*,[13] respectively. Russian strain is referred to as RUS by Snell *et al.*[11] For each strain, a clonal culture was established for experimentation.

Mating Assay

Experimental procedure was the same as described in Hagiwara *et al.*[9] Each strain was cultured in total darkness. Culture temperature was 15, 20 and 25℃ and salinity was 22 ppt. Rotifer populations were cultured at each temperature for more than one week before experiments. *Nannochloropsis oculata* was fed to rotifers daily to maintain 7 million cells/ml.

Mating behavior of rotifers was recorded male contact, circling, and penile attachment (copulation). Two males and one female collected from each culture were transferred into 15μl of seawater. This small test volume was necessary to promote frequent contact between males and the female.

The mating assays consisted of intrastrain matings and interstrain matings, all conducted at male culture temperature. In the intrastrain matings, the following three experiments were done: 1) using males and females cultured at same temperature (15, 20 or 25℃), 2) using males cultured at 20℃ and females at 15, 20℃ or 25℃, 3) using males cultured at 15, 20 or 25℃ and females at 20℃. Males and females were tested in 9 combinations for each strain. The interstrain assay was also done at each temperature. In every combination, number of male circlings after 20 female contacts were counted, as well as the number of subsequent copulations. The above observations were repeated 6 times for each pairing with males and females used only once.

A chi square contingency test was employed to identify significant differences in how males reacted to females of their own strain and different strains.

Anti-MRP Binding Assay

Lyophilized anti-MRP was prepared from the MRP of Russian strain.[11] The activity of anti-MRP does not change by lyophilization. The procedure for anti-MRP binding assay is the same as described by Kotani *et al.*[10] This antibody (80 μg IgG) was dissolved in 100 μl of HMNK buffer (50mM HEPES, 36mM NaCl, 1mM HCl, 0.1mM MgSO4, pH 7.4). The solution was added to 100 μl of DMSO containing 1mg of biotin (succinimidyl 6- (biotinamido) hexanoate (biotinamidocaproate, N-hydroxysuccinimidyl ester), Molecular Probes Inc.). The mixture was shaken at 150 rpm for 90 min to promote biotin binding to the antibody. To remove unbound biotin, the mixture was placed in a test tube with a 30,000 MW cut off filter (Centricon-30, Amicon Inc.) and centrifuged at 2,500 rpm for 60 min. Biotinylated anti-MRP was retained out the filter and washed off with 100 μl of HMNK buffer.

Rotifer females carrying parthenogenetic eggs were collected from culture and placed in 500 μl of HMNK buffer along with *N. oculata* (7 million cells/ml). After 4~6h, 20 neonate females were isolated and exposed to 50 μl biotinylated anti-MRP-HMNK solution for 1 h to allow binding of antibody to the MRP. Then neonate females were washed twice by transferring to 500 μl of buffer HMNK. After washing, the females were exposed for 1 h to 500 μl of buffer HMNK containing 50 μl of a 1 : 20 solution of avidin-labeled, 1 μm fluorescein-containing latex beads (Fluospheres, Molecular Probes Inc.). Avidin bound to biotin on the anti-MRP, fluorescently labeling MRP sites on females. Females were washed twice again by transferring them to 500 μl of HMNK buffer, then transferred to a slide for observation with an epifluorescent microscope (BH2-RFL, Olympus Inc.) at 100x. Images were captured by a Macintosh Quadra 650 computer using an Olympus CCD camera (model FCD-725). Quantification of fluorescence was performed using NIH Image according to Snell and Morris.[14] A background measurement from the body of each rotifer was subtracted from the coronal measurement. A one-way ANOVA to test the influence of temperature on coronal fluorescence and Tukey's HSD test were employed to identify significant differences among groups cultured at different temperatures.

Results

Mating assays

The percent of male-female encounters resulting in mating attempts of Russian and Tokyo strains were comparatively high values ranging between 27-53%, while that of German

Table 1. Influence of different temperatures on mating attempts and copulation between males and females of three *B. plicatilis* strains. Chi-square tests the hypothesis that mating attempts and copulations in each combination occur with equal frequency.

Combination	% mating attempts			% copulations		
	Russia	Tokyo	Germany	Russia	Tokyo	Germany
15℃ ♀ ×20℃ ♂	34.8	49.9	1.7	16.4	11.7	0.0
20℃ ♀ ×20℃ ♂	46.4	49.1	5.0	10.0	7.1	1.7
25℃ ♀ ×20℃ ♂	27.5	52.6	0.0	3.3	9.2	0.0
χ^2 value	9.1	<0.1	4.0	4.3	1.8	2.0
p value	0.010	0.988	0.137	0.115	0.400	0.365
20℃ ♀ ×15℃ ♂	38.3	26.7	3.3	5.0	1.7	0.0
20℃ ♀ ×20℃ ♂	46.4	49.1	5.0	10.0	7.1	1.7
20℃ ♀ ×25℃ ♂	47.5	45.6	1.7	6.7	6.9	0.0
χ^2 value	2.4	14.6	1.3	2.4	4.3	2.0
p value	0.301	0.001	0.530	0.303	0.114	0.365

strain remained very low (0–5.0%) (Table 1). Culture temperature of males or females had little effect on mating. Males of Russian strain showed the highest frequency of mating attempts against females cultured at 25℃, but mating attempts of Tokyo and German strain males did not differ significantly with females cultured at 15, 20 or 25℃. In experiments using males cultured at 15, 20 and 25℃ and females at 20℃, Tokyo strain males cultured at 15℃ had a lower frequency of mating (26.7%, $p<0.01$). Russian and German strain males cultured at different temperatures, however, recognized females at equal frequencies. The frequency of copulations per encounter did not significantly vary among rearing temperatures.

Table 2 shows the frequency of mating attempts and copulations in encounters in interstrain mating assays. Among nine combinations of treatments (three temperatures for male culture times *B. plicatilis* strain females), significant differences in mating attempts were found in the five treatments. These include, Russia(♂) vs Russia(♀), Russia(♂) vs Tokyo(♀), Germany(♂) vs Russia(♀), Germany(♂) vs Tokyo(♀) and Germany(♂) vs Germany(♀). No consistent trend was found between male culture temperature and mating attempts. For example, Russian males cultured at 15℃ showed lowest mating attempts with Russian females, while they attempted mating at the highest frequency against Tokyo females. Under the three different temperatures, Tokyo strain males attempted mating at equal frequencies against females of three rotifer strains.

Anti-MRP Binding Assay

When culture temperature of females was changed, significant differences in anti-MRP binding and subsequent fluorescence intensity was observed only with German strain (Fig. 1). The fluorescence intensity of German strain was highest at 15℃ (mean±SD=29.4±13.7, df=2, F=6.259, $p<0.01$).

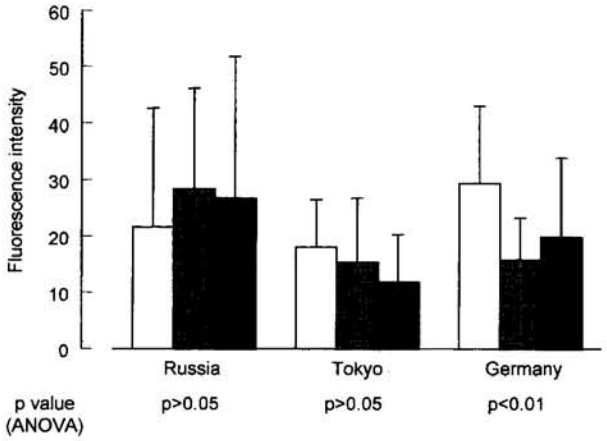

Fig. 1. Anti-MRP binding to females of three strains at three temperatures. White, gray and black columns represent coronal fluorescence at 15, 20, 25℃, respectively. Variation is represented by vertical bars indicating one standard deviation (n=20 for fluorescence at corona).

Table 2. Intra-and interstrain mating attempts and copulations in three *Brachionus plicatilis* strains at three temperatures. Chi-square tests the hypothesis that self-and cross-mating attempts and copulations at all temperatures occur with equal frequency.

Female	Temperature (℃) Male	% mating attempts			% copulations		
		Russia	Tokyo	Germany	Russia	Tokyo	Germany
Russia	15	28.3	2.5	4.2	3.3	0.0	0.0
	20	46.4	0.8	0.0	10.0	0.0	0.0
	25	38.3	0.8	0.0	10.8	0.0	0.0
	χ^2 value	8.2	1.6	10.1	4.3	–	–
	p value	0.017	0.444	0.006	0.117	–	–
Tokyo	15	60.0	44.8	50.8	6.7	6.8	1.7
	20	31.7	49.1	15.0	0.0	7.1	0.0
	25	46.0	38.3	21.7	0.8	2.5	2.5
	χ^2 value	19.4	3.2	42.2	12.9	2.5	2.8
	p value	<0.001	0.197	<0.001	0.002	0.281	0.242
Germany	15	24.2	1.7	0.0	4.2	0.8	0.0
	20	23.3	0.0	5.0	0.0	0.0	1.7
	25	32.0	1.7	0.8	1.7	0.8	0.0
	χ^2 value	2.1	2.0	9.0	5.5	1.0	4.0
	p value	0.345	0.364	0.011	0.063	0.605	0.134

Discussion

Sexual and asexual reproduction of monogonont rotifers is regulated by both internal and external factors.[15-17] Resting eggs are the final product of sexual reproduction that include: mictic female production by amictic females, male production by unfertilized mictic females, mating, fertilization and resting egg production by fertilized mictic females. Hagiwara *et al.*[18] demonstrated that temperature and salinity of rotifer cultures can have large effects on resting egg. They employed male swimming speed as an index of mating frequency. In this study, we also observed that the frequencies of attempted mating and copulation can also be affected by temperature. But the temperature effect on male mate recognition was weak and varied among strains. Recognition of females by Russian and German males were temperature dependent, although such a trend was not observed for Tokyo strain (Table 2). Theoretically, temperature could affect the MRP synthesis as well as its distribution density on the female's body. Similar effects might be expected for the male's receptor. The temperature dependence of male mate recognition of the Russian strain was more related to culture temperature of females rather than males (Table 1). Although we hypothesized that such a difference might be caused by the temperature effects on MRP synthesis and abundance, the anti-MRP binding assays did not confirm this.

Differences in mating frequencies from interstrain crossing have been well explained by the degree of anti-MRP binding with MRP.[8,10] We observed no indication that temperature dependent frequencies of attempted matings was due to modification of MRP structure or abundance. The German strain in this study showed less mating frequencies in intrastrain crossing as was observed by Kotani *et al.*[10] For *B. rotundiformis* Hawaiian strain, Hagiwara *et al.*[19] observed low fertilization at 4 ppt salinity even though mixis induction and male production was active in the culture. A possible hypothesis to explain these observations is that the swimming behavior of male and female rotifers is affected by temperature, which results in different frequencies of encounter and subsequent copulation. Further study is needed to clarify the mechanism of how environmental conditions affect mating in monogonant rotifers.

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References

1) J. J. Gilbert: Contact chemoreception, mating behavior, and sexual isolation in the rotifer genus *Brachionus*. *J. Exp. Biol.*, **40**, 625–641 (1964).
2) T. W. Snell and C. A. Hawkinson: Behavioral reproductive isolation among populations of the rotifer *Brachionus plicatilis*. *Evolution*, **37**, 1294–1305 (1983).
3) P. Clement, E. W. Wurdak and J. Amsellem: Behavior and ultrastructure of sensory organs in rotifers. *Hydrobiologia*, **104**, 89–130 (1983).
4) T. W. Snell, M. J. Childress and B. C. Winkler: Characteristics of the mate recognition factor in the rotifer *Brachionus plicatilis*. *Comp. Biochem. Physiol.*, 89A, 481–485 (1988).
5) T. W. Snell and M. A. Nacionales: Sex pheromone communication in *Brachionus plicatilis* (Rotifera). *Comp. Biochem. Physiol.*, **97A**, 211–216 (1990).
6) T. W. Snell, P. D. Morris and G. Cecchine: Localization of the mate recognition pheromone in *Brachionus plicatilis* O. F. Müller (Rotifera) by fluorescent labeling with lectins. *J. Exp. Mar. Biol. Ecol.*, **165**, 225–235 (1993).
7) A. Gómez and M. Serra: Behavioral reproductive isolation among sympatric strains of *Brachionus plicatilis* Müller 1786: insights into the status of this taxonomic species. *Hydrobiologia*, **313/314**, 111–119 (1995).
8) R. Rico-Martinez and T. W. Snell: Male discrimination of female *Brachionus plicatilis* Müller and *Brachionus rotundiformis* Tschugunoff (Rotifera). *J. Exp. Mar. Biol. Ecol.*, **190**, 39–49 (1995).
9) A. Hagiwara, T. Kotani, T. W. Snell, M. Assava-Aree and K. Hirayama: Morphology, genetics and mating behavior of small tropical marine *Brachionus* strains (Rotifera). *J. Exp. Mar. Biol. Ecol.*, **194**, 25–37(1995)
10) T. Kotani, A. Hagiwara and T. W. Snell: Genetic variation among marine *Brachionus* strains and function of mate recognition pheromone (MRP). *Hydrobiologia*, **358**, 105–112 (1997).
11) T. W. Snell, R. Rico-Martínez, L. S. Kelly and T. E. Battle: Identification of a sex pheromone from a rotifer. *Mar. Biol.*, **123**, 347–353 (1995).
12) R. Rico-Martínez and T. W. Snell: Comparative binding of antibody to a mate recognition pheromone on female *Brachionus plicatilis* and *Brachionus rotundiformis* (Rotifera). *Hydrobiologia*, **358**, 71–76 (1997).
13) Y. Fu, A. Hagiwara and K. Hirayama: Crossing between seven strains of the rotifer *Brachionus plicatilis*. *Nippon*

Suisan Gakkaishi, **59**, 2009–2016 (1993).
14) T. W. Snell and P. D. Morris: Sexual communication in copepods and rotifers. *Hydrobiologia,* **255/256**, 109–116 (1993).
15) R. Pourriot and T. W. Snell: Resting eggs in rotifers. *Hydrobiologia,* **104**, 213–224 (1983).
16) E. Lubzens: Raising rotifers for use in aquaculture. *Hydrobiologia,* **147**, 245–255 (1987).
17) A. Hagiwara: Practical use of marine rotifer cysts. *The Israeli Journal of Aquaculture-Bamidgeh,* **46**, 13–21 (1994).
18) A. Hagiwara, A. Hino and R. Hirano: Effects of temperature and chlorinity on resting egg formation in the rotifer *Brachionus plicatilis. Nippon Suisan Gakkaishi,* **56**, 569–575 (1988).
19) A. Hagiwara, C.-S. Lee, G. Miyamoto and A. Hino: Resting egg formation and hatching of the S-type rotifer *Brachionus plicatilis* in different salinities. *Mar. Biol.*, **103**, 327–332 (1989).

異なる温度でのシオミズツボワムシ *Brachionus plicatilis* Müller の配偶者認知

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海産ワムシ類の性フェロモン（以下 MRP）分子構造は種間，株間で異なっており，これが要因となって，交尾頻度の変化や生殖的隔離が生じる。しかし，環境がMRPの性状にどのような影響を与えるかについては明らかでない。本研究では，シオミズツボワムシ（以下ワムシ）の生殖的隔離に対する温度の影響を調べることを目的とした。

ロシア株，ドイツ株，東京株の3株のワムシを用いた。ワムシ3株間で9通りの雌雄の組み合わせを作り，それぞれ3段階の温度（15，20，25℃）での交尾頻度を比較した。同じ株の雌雄について，あらかじめ異なる温度（15，20，25℃）で培養後，共存させた場合についても検討を行った。同時に MRP 抗体の反応度を求めることにより，各温度下での雌ワムシ繊毛冠上 MRP 分布密度を比較した。

ロシア株の♂と♀，ロシア株♂と東京株♀，ドイツ株♂とすべての株の♀との交尾頻度は温度間で有意に異なり，ロシア株内では，雄は20℃で培養した雌に対して最も高い交尾頻度を示した。同様に，ドイツ株では20℃以下で培養した雌に対してのみ交尾が起こった。この時，雄の培養温度に関わらず同様の頻度で雌（20℃で培養）に交尾した。一方，東京株では15℃で培養した雄は雌に対する交尾頻度が低くなった。各温度での MRP 抗体の反応度は各々の温度による雄の交尾頻度とは相関が無かった。