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Ecological factors affecting gene flow in the *Brachionus plicatilis* complex (Rotifera)

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Abstract We investigated how adaptation to salinity and temperature acts as reproductive barriers in three sympatric species from the *Brachionus plicatilis* species complex. These species co-occur in a salt marsh in Spain, and a previous electrophoretic study of variation revealed no hybrids between them. A factorial experiment was designed to test for differences in population growth rates and patterns of bisexual reproduction. The design combined representative strains from each species in different salinity and temperature conditions, representing the range over which these rotifers are found in their natural environment. We found differences in the growth response of the three species to both factors and in the pattern of bisexual reproduction. These differences help to explain patterns of succession observed in the field. We conclude that these ecological factors, together with mate recognition systems, account for the absence of gene flow in these sympatric species.

Key words Rotifers · *Brachionus plicatilis* · Bisexual reproduction · Ecological specialization · Mate recognition system

Introduction

Monogonont rotifers are common planktonic invertebrates with a cyclically parthenogenetic life cycle (Bell 1982; Wallace and Snell 1991). Parthenogenetic reproduction by asexual (amictic) females in the absence of males (amictic phase) predominates in this life cycle, but there are periods where both parthenogenetic and bisexual reproduction (mictic phase) occur simultaneously. In the mictic phase, both sexual (mictic) females and males appear in the population, and bisexual reproduction (mixis) takes place. The presence of bisexual reproduction makes it possible to use mating behavior as a criterion to recognize rotifer species (Snell and Hawkinson 1983; Snell 1989). In fact, this criterion helped to distinguish sibling species within the species complex of *Brachionus plicatilis* O.F. Müller 1786 (Snell and Hawkinson 1983; Gómez and Serra 1995; Rico-Martínez and Snell 1995).

Within the context of the recognition species concept, the specific mate recognition system (SMRS) is the central defining property of a species (Paterson 1985; Lambert et al. 1987). The SMRS consists of the signal-receptor mechanisms between males and females that operate during mating. Mate recognition is only relevant if sexual individuals co-occur; thus, ecological factors affecting co-occurrence such as activity patterns of males and females, timing of sexual reproduction, and restriction of organisms to specific habitats (Paterson 1993) must be taken into account in any study of SMRS, as they may act as reproductive barriers. In brachionid rotifers, a central element of mate recognition is a sex pheromone – a 29 kD glycoprotein in *B. plicatilis* – located on the female body surface, which is required to elicit male mating behavior (Snell et al. 1995). Furthermore, in rotifers habitat preferences or ecological tolerances may indirectly determine when and where bisexual reproduction can occur, and environmental factors directly affect the timing and extent of bisexual reproduction. Bisexual reproduction – usually restricted to a few weeks during the annual cycle – is strongly dependent on both population density and environmental conditions (Gilbert 1977; Pourriot and Snell 1983; Lubzens et al. 1980, 1985; Hagiwara et al. 1988a, b; Snell and Boyer 1988; Carmona et al. 1994, 1995).

Brachionus plicatilis s.l. (*sensu lato*) provides a good model to investigate the specific mate recognition systems of rotifers, as it has been recently recognized as a complex of sibling species (Gómez et al. 1995; Segers 1995), with two named species (*B. rotundiformis* and *B. plicatilis s.s.*, *sensu stricto*, Segers 1995). Moreover, there is evidence for the occurrence of more species in

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the complex based on allozyme and mating behavior data (Snell and Hawkinson 1983; Gómez et al. 1995; Gómez and Serra 1995; Rico-Martínez and Snell 1995; Snell et al. 1995; Gómez and Snell 1996). In contrast, little is known about the role of ecological factors as reproductive barriers between the species of the *B. plicatilis* complex. Unfortunately, the extensive ecological information in the literature on this taxon fails to provide the required data to address this question as the presence of sibling species in *B. plicatilis* was not recognized until recently. Nevertheless, according to previous empirical studies, *B. rotundiformis*, grows better in warmer temperatures and *B. plicatilis s.s.* in colder conditions (e.g., Ito et al. 1981). This differentiation may determine in which season each species can be present. In addition, temperature and salinity were strongly correlated with the presence of the three species of the *B. plicatilis* complex in Torreblanca Marsh, a coastal marsh in Spain (Gómez et al. 1995). On the other hand, genetic variation in the mictic ratio and resting egg production in different strains of *B. plicatilis s.l.* depending on the environmental conditions has also been found (Snell and Hoff 1985; Hagiwara et al. 1988a, b; Carmona et al. 1994).

In this study, we focus on a well studied system, the *B. plicatilis* species complex (Rotifera) in Torreblanca Marsh (Gómez et al. 1995). According to previous work (Gómez et al. 1995), three species, recognizable through allozyme markers and morphological traits, from this species complex co-occur in this marsh and are involved in a seasonal succession: *B. plicatilis s.s.* (the L clonal group), the largest species, and two species that fit morphologically into *B. rotundiformis*, a small species (SS clonal group) and an intermediate-sized species (SM clonal group). Allozyme data suggests that no gene flow exists between these species in sympatry. In fact, cross-mating experiments showed that mating behavior can partially account for the absence of interbreeding among these sympatric species (Gómez and Serra 1995). Given that the spatial and temporal distributions of these species suggest ecological divergence among them (Gómez et al. 1995), a role of ecological factors as reproductive barriers can be hypothesized. In order to test this hypothesis, we determined the role of salinity and temperature, two parameters correlated to the differential abundance of these species (Gómez et al. 1995), in the segregation of bisexual periods among species. To this end we addressed differences in population growth rate and bisexual reproduction investment of laboratory strains.

Materials and methods

We studied three sympatric strains of the *B. plicatilis* complex, L1, SM2 and SS2, originally isolated from single amictic females collected in Torreblanca marsh in 1992–1993 (Gómez et al. 1995). These strains are representative from common genotypes in their species. L1 is a *B. plicatilis s.s.* strain, SS2 is a strain from the *B. rotundiformis* SS species and SM2 is from the *B. rotundiformis* SM species. All strains have been batch-cultured since then in our laboratory on *Tetraselmis suecica* at 12 g/l salinity, 25°C and constant illumination. Saline water was made with commercial seasalts (Instant Ocean) and fertilized with $f/2$ solution (Guillard and Ryther 1962) just before algal inoculation. In the experiments, growth medium was the alga *T. suecica*, cultured in the experimental salinity and acclimatized to experimental temperature.

In a preliminary life-table experiment, we explored the strains' tolerance of low temperature (10°C), using 48 neonate females from each strain placed in 0.5 ml growth medium at 10 g/l salinity in 24-well culture plates. Females were observed daily until their death, and offspring, if produced, were counted and removed. Food was provided in excess (10^6 cells of *T. suecica*/ml) at the beginning of the experiment, so addition of extra food was not required during the experiment.

The main experiment had a factorial design of salinities (9, 13 and 22 g/l) and temperatures (15, 20 and 25°C) representative of those in the field (Gómez et al. 1995). We studied effects of these factors on growth rates and mictic ratios in each strain. For pre-experimental and experimental cultures, algal density was adjusted to 10^6 cells of *T. suecica*/ml, which provided rotifers with an excess of food. Each rotifer strain was acclimatized to the experimental conditions for at least a week before the beginning of the experiments. Pre-experimental cultures were set up placing 3–10 egg-bearing females acclimatized to the experimental salinity and temperature in 500 ml glass bottles with growth medium for 2–10 days. Density in pre-experimental cultures was always under 0.15 females/ml which is the density threshold for induction of mixis in *B. plicatilis s.s.* (Russian strain) reported by Snell and Boyer (1988). Preliminary observations revealed that at this low density our strains did not show sexual reproduction. In this way, we made sure that females used to start the experiment were reproducing parthenogenetically. The experiment began by placing three to five egg-bearing females of a given strain from the pre-experimental culture in 250 ml of growth medium at the experimental salinity and temperature. In total, 27 treatments were set up (3 strains in 3 temperature conditions in 3 salinities) with two replicate cultures each.

We sampled 5–10 ml of each experimental culture every day (at temperatures 20 and 25°C) or every other day (15°C). The sampling volume was not returned to the experimental culture, except for those instances in which less than two rotifers were found in the sample. If available, 48 living females from the sample were isolated in growth medium and incubated in the experimental salinity and temperature until offspring appeared, in order to assess their reproductive mode. Amictic females produce daughters and mictic females produce males, if not fertilized, or resting eggs, if fertilized. The remainder of the sample was fixed using Lugol's iodine and population densities were estimated by counting the females. Culture sampling proceeded until enough data were collected to estimate growth rates and mictic ratios.

We estimated the per capita growth rates (r) by least square regression using the equation $ln\ N_t = ln\ N_0 + rt$ for the growth period in which $ln\ N_t$ changed linearly. This guarantees that density effects (e.g., competition or density-dependent variation in mictic ratios) are negligible. In cyclical parthenogens, r is not an accurate indication of the individual ability to reproduce under specific conditions, as r also reflects the growth depression due to mictic female production. But, precisely because it takes this into account, our estimate of r is appropriate for knowing the conditions in which a population can grow; that is, when total fecundity investment exceeds mixis investment. Those cultures in which population density did not reach 1 female/ml were considered not to grow. We performed an ANOVA on growth rate using as factors strain, temperature and salinity conditions.

In order to test the correspondence between laboratory growth rates and field observations, the presence of each species found in Torreblanca Marsh (Poza Sur pond) in a given sampling date from the sampling season 1992–1993 (Gómez et al. 1995) was predicted on the basis of the growth rates of the strains estimated in the laboratory in this study. The estimation was made by a two-way linear interpolation for the salinity and temperature in the field from the experimental r values. Presence of a species in a given sampling date was predicted if its estimated r was positive.

In each sample, mictic ratios were calculated by dividing the number of mictic females by the total number of females whose reproductive status was assessed. The average mictic ratio over the growth period was used as a measure of the investment in mixis of a strain in a given combination of temperature and salinity. In order to compare the patterns of bisexual reproduction, we estimated, using regression analysis, lowest density threshold for mixis induction as the population density at which the mixis ratio reached 5%. For each strain, we calculated ANOVAs on density threshold for mixis and arcsin transformed average mictic ratio, using as factors salinity and temperature. In the analyses of average mictic ratio, we added as a covariate the average density of rotifers in each culture during the period in which mixis was estimated. As covariation with density is different for each strain, we conducted a separate ANOVA for each one.

To evaluate how mictic patterns and adaptation to different salinity and temperature affect mating patterns, we estimated the average percentage of heterogamic male-female encounters. We took into consideration the density of females of each strain and the mictic ratio during the experiment to estimate the average number of mictic females for each combination of salinity and temperature. We assumed that male density is proportional to average number of mictic females belonging to the same strain. Total proportion of heterogamic male-female encounters (Hy) was estimated as

$$Hy = 1–(Ho_L + Ho_{SM} + Ho_{SS})$$

where Ho is the number of encounters of males from one strain with females from the same strain, which, following probabilistic rules of random encounter (Snell and Garman 1986), is calculated as the squared proportion of females of this strain in the population. The maximum possible number of heterogamic encounters occurs when mictic females are equally frequent in the population (that is, 0.33 each), which represent an $Hy = 0.67$. Thus, we can evaluate, for each condition explored in our experiment, the percentage of reduction in number of heterogamic encounters between males and females over the maximum possible number of such encounters.

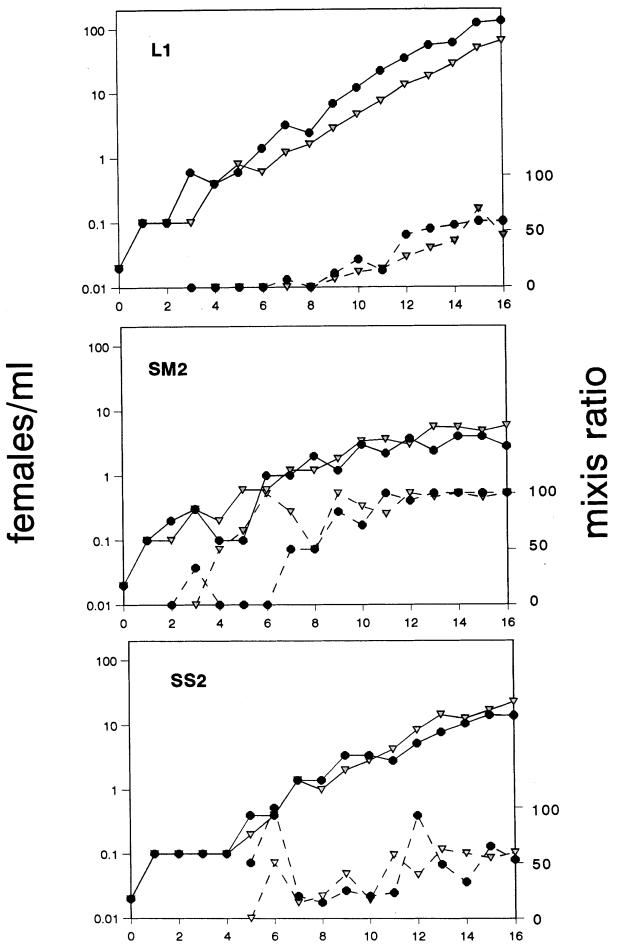

Fig. 1 Example of the results of the factorial experiment. Variation in population densities (*continuous line*) and mictic ratios (%, *dashed line*) for L1, SM2, and SS2 strains of *Brachionus plicatilis* at 20°C and 13 g/l. The different *symbols* represent different replicates

Results

Growth rates

In the life-table experiment, no growth occurred for SS2 and SM2 strains at 10°C. SS2 females ($n = 48$) died within 8 days of the onset of the experiment, leaving no offspring. All SM2 females ($n = 48$) died within 22 days, and no offspring were produced. The L1 strain tolerated 10°C and individuals were able to reproduce with an r of 0.1 day^{-1}.

An example of the results for the factorial experiment is shown in Fig. 1. ANOVA showed that all main effects and their 1st- and 2nd- order interaction were highly significant. Temperature was responsible for most of the variation in r (Table 1). Growth rates for all strains were maximum at higher temperatures (Fig. 2). However, a differential response of the strains existed in the rate at which growth rates increase with temperature. The linear increase in growth rates with temperature was at a maximum for SS2 (0.12), intermediate for SM2 (0.10) and low for L1 (0.09). At the lowest temperature (15°C), SS2 was unable to grow irrespective of salinity conditions. In SM2 no growth occurred at 15°C and 22 g/l salinity. At higher temperatures the growth rate of each of the strains tended to decrease with salinity, which was more pronounced in strain SM2.

Table 1 ANOVA results of the three factorial fixed effects (strains, salinity and temperature) on the growth rate (r)

Factor	*df*	*F*
Strain	2,30	23.72**
Salinity	2,30	44.47**
Temperature	2,30	1169.40**
Strain × salinity	4,30	6.92**
Strain × temperature	4,30	14.30**
Salinity × temperature	4,30	12.59**
Strain × salinity × temperature	8,30	4.90**

**$P \leq 0.001$

The correlation between our prediction of presence and actual presence of each species in the field in each sampling date was statistically significant (32 agreements for a total of 48 observed-predicted pairings, $\phi_2 = 0.37$, $P < 0.05$, using χ^2 corrected for continuity; Zar 1987). The majority of disagreements (14 out of 16 pairings) were due to absences in the field predicted as presences.

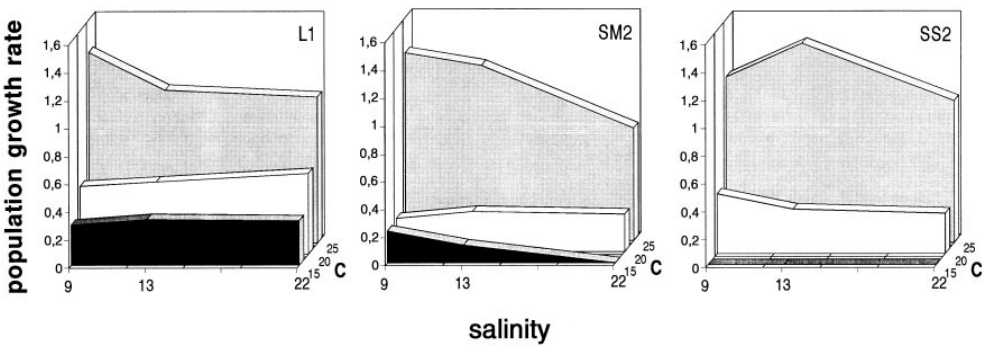

Fig. 2 Population growth rates (r) of the three strains L1, SM2 and SS2 at the experimental salinity and temperature conditions. Values shown are averages of the two replicate cultures

Induction of mixis

Mictic ratio was not linearly correlated with density, but a logistic function fitted best. This is to be expected if the mictic ratio values are constrained, as happened in this experiment, since mixis ratios ranged from very low values (near 0) to very high ones (close to 1). Therefore, density thresholds for mixis were calculated using a logistic function regression of mictic ratio against population density, whose parameters were estimated from the experimental data for each combination of temperature, salinity, strain and replicate. Average density thresholds for mixis induction (i.e. the population density at which mictic ratio reaches 5%) were very different for L1, SM2 and SS2 strains (Table 2). SS2 showed the highest density threshold, while L1 and SM2 strains had lower, and similar, thresholds. This indicates that, in the conditions studied here, mixis is induced at lower densities in SM2 and L1 than in SS2 (Table 2).

In strain L1, salinity effect on average mictic ratio was highly significant (Table 3; Fig. 3). Average mictic ratio in this strain decreased with increasing salinity and mixis was highest at the lowest salinity tested (9 g/l). The density threshold for mixis in strain L1 increased with salinity (Table 3; Fig. 3). This can explain, at least partially, why mixis investment is higher at lower salinities in this strain. For mictic ratio, although temperature main effect was not significant, the effect of temperature depended on salinity. Thus, mictic ratio increased with temperature at low salinity, but strongly decreased with temperature at high salinity. An interaction effect in the density threshold was also found, mainly due to the increase of the threshold at high salinity and temperature.

Table 2 Average density threshold for mixis and average mictic ratio (averaged for all conditions) in the three strains studied. All temperatures are included in the averages. If the temperature in which the strain SS2 did not grow (20°C) was not included in average computation, results are not qualitatively changed

Strain	5% Density threshold (females/ml)	SD	Range of density threshold	Average mictic ratio
L1	10.9	4.8	1.0– 85.3	32.3
SM2	8.8	3.0	0.4– 36.6	42.5
SS2	86.6	24.3	5.2–185.2	12.7

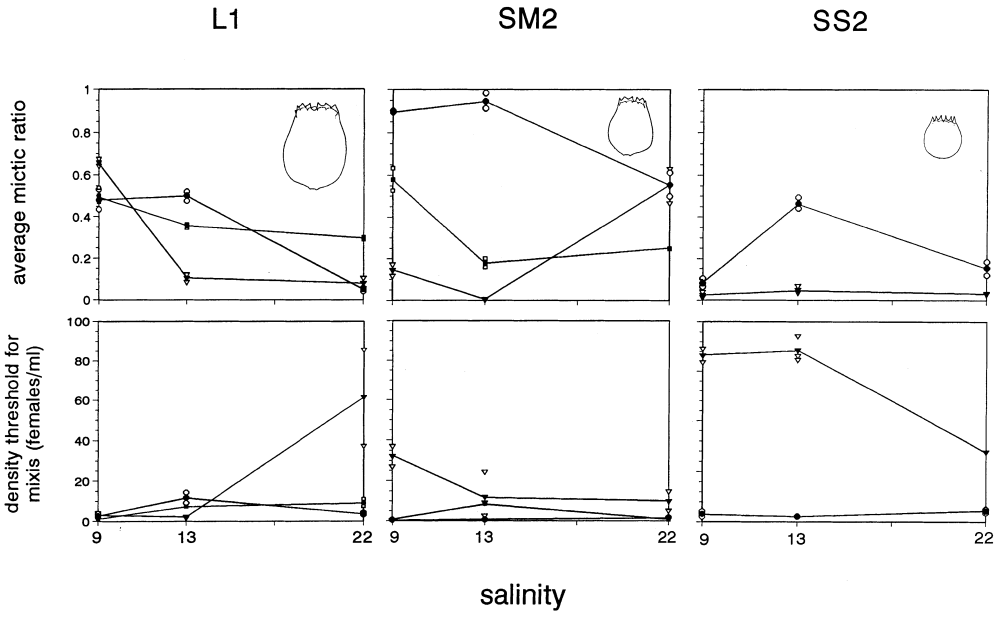

Fig. 3 Relationship of average mictic ratio and density threshold for 5% mixis with temperature and salinity in the three strains studied. *Solid symbols* represent averages of the replicates. The two replicates are shown as *blank symbols*. In the case of mictic ratio the replicates represent average values corrected for density for each culture. *Squares* represent 15°C, *circles* 20°C, and *triangles* 25°C. *Insets* show camera lucida profiles of the lorica or caparace of adult females of each strain

Table 3 ANOVA table of F values and degrees of freedom for density thresholds for mixis and arcsine transformed mictic ratios for each strain. (S salinity, T temperature)

Strain	Source of variation	Density threshold		Mictic ratio	
		df	*F*	*df*	*F*
L1	T	2,9	3.81 n.s.	2,9	0.73 n.s.
	S	2,9	6.32*	2,9	36.48**
	T × S	4,9	6.12*	4,9	13.53**
SM2	T	2,6	7.31*	2,9	66.99**
	S	2,6	0.93 n.s.	2,9	5.17*
	T × S	4,6	2.05 n.s.	4,9	17.59**
SS2	T	1,5	415.24**	1,6	26.33*
	S	2,5	23.86*	2,6	44.59**
	T × S	2,5	28.26*	2,6	38.60**

*$P \leq 0.05$, **$P \leq 0.001$, n.s. not significant

The lowest density threshold occurred at the lowest temperature and salinity tested (15°C and 9 g/l).

In strain SM2, salinity, temperature, and their interaction affected mictic ratio significantly (Table 3; Fig 3). Mictic ratio was highest at 20°C and 13 g/l, even when corrected for density. Density threshold for mixis was significantly affected only by temperature and was highest at 25°C.

In strain SS2, temperature, salinity and their interaction affected average mictic ratio and density threshold (Table 3). SS2 mictic ratio was highest at 13 g/l salinity and 20°C, the lowest temperature in which this strain was able to grow in these experiments (Fig. 3). Average patterns of mixis can be explained in part by the low density threshold for this strain at 20°C and 13 g/l salinity.

In the estimation of heterogamic male-female encounters (Hy) we found, on average, a 34% reduction of heterogamic encounters from the maximum number possible. This reduction is caused by the different density responses and mixis patterns of each strain.

Discussion

The study of speciation in rotifers is interesting as their life cycles have several distinctive features. First, rotifers opportunistically exploit temporary habitats until less opportunistic species, such as cladocerans or copepods, outcompete them (Allan 1976; Gilbert 1985), or their occurrence is seasonally constrained. Thus, bisexual reproduction should occur before the habitat becomes unsuitable. Second, over the course of a population growth cycle, bisexual reproduction is infrequent compared with parthenogenesis, and to be effective must be a synchronized event affecting a large proportion of the population. Third, visual components of mating behavior are unimportant in rotifers since their sensory systems are primarily chemical and mechanical (Clément et al. 1983; Snell and Morris 1993). This fact is thought to promote cryptic speciation with the consequent formation of sibling species (Knowlton 1993).

Mating behavior has been used to define the limits to gene flow in rotifers (Snell and Hawkinson 1983; Snell 1989; Gómez et al. 1995), particularly because a correlation between genetically distinct clonal groups in the field and mating pattern in the lab has been found (Gómez and Serra 1995), with few heterogamic mating attempts. However, some mating attempts were recorded for co-occurring species although there is no evidence for hybridization in the field. Hence, there is a putative role for ecological factors as reproductive barriers.

Our experimental results predict to a large extent the field patterns of the three species of the complex *B. plicatilis*. The few disagreements found, due to absences of a species in the field predicted as presences, may be due to the overestimation of growth rates in laboratory conditions due to excess food, absence of predators, and absence of interspecific competitors. Seasonal succession and temporal niche segregation in the field can be partially explained by a differential response of the strains to salinity and temperature. These results indicate that ecological adaptation and mictic responses to density, salinity and temperature join the specific mate recognition systems in explaining absence of gene flow between sympatric species from the *B. plicatilis* complex. These two factors – ecological adaptation and mictic responses – determine (1) when density thresholds for sexual reproduction can be surpassed, through a determination of population growth rates, and (2) how propensity towards bisexual reproduction is affected by ecological conditions other than population density.

L strains belonging to *B. plicatilis s.s.* are adapted to lower temperatures than smaller strains belonging to *B. rotundiformis* SM and *B. rotundiformis* SS; e.g., *B. plicatilis s.s.* tolerates lower temperatures (10°C). The evidence in the literature (Table 4) and field observations (Gómez et al. 1995) support this ecological specialization, which involves not only that maximum growth rates for *B. plicatilis s.s.* occur at lower temperatures, but that slope of growth rate on temperature is lower in this species (as derived from Miracle and Serra 1989). Moreover, in competition experiments, *B. plicatilis s.s.* outcompetes *B. rotundiformis* under 20°C and the reverse is true over 27°C (Hagiwara et al. 1995). The two species currently included within *B. rotundiformis* can also be distinguished by their temperature tolerances, as *B. rotundiformis* SM is able to grow at 15°C but *B. rotundiformis* SS is not. Salinity responses of these two species in the lab are also consistent with field results (Gómez et al. 1995) and data on other strains from this complex (Table 4). Thus, *B. rotundiformis* SM seems to be adapted to low salinity, *B. rotundiformis* SS to high salinity, and *B. plicatilis s.s.* to low to moderate salinity (Table 4). The diverse results among studies for optimum salinity for *B. rotundiformis* further support that more than one species is included in this taxon.

Differences in the timing and pattern of bisexual reproduction of *B. plicatilis s.s.* and *B. rotundiformis* (SS and/or SM) have been observed in Torreblanca marsh (Carmona et al. 1995). *B. plicatilis s.s.* had a low density

Table 4 Population growth rates and mixis investments of strains from the *Brachionus plicatilis* complex growing in different salinity and temperature conditions. Temperature and salinity when growth rates and mictic rates were maximum are given. The *symbols* – and + indicate that the mixis or growth parameter attained the highest value at the minimum or maximum values used for the environmental parameters. If not explicitly stated, the taxonomic status of each strain was inferred from the morphotype or morphological features of each strain as given by the authors. In the case of SS and SM *B. rotundiformis* the strains were distinguished on the basis of their allozyme patterns (Gómez et al. 1995)

Species	Salinity (g/l)		Temperature (°C)		Source[a]
	Growth	Mixis	Growth	Mixis	
B. rotundiformis					
	8^{-}	32^{+}	–	28	(3, 4)
	40^{+}	25	30	25	(13)
	2	–	35	–	(11)
	–	–	33–35	–	(7)
B. rotundiformis (*SM*)					
	9^{-}	–	30^{+}	–	(10)
	9^{-}	9^{-}	25^{+}	20	(This study)
B. rotundiformis (*SS*)					
	$12–24^{+}$	–	30^{+}	–	(10)
	13	13	25^{+}	20	(This study)
B. plicatilis					
	10	4	–	–	(8, 9)
	12	–	30^{+}	–	(10)
	–	–	27–28	–	(7)
	–	10^{-}	–	15^{-}	(5, 6)
	–	10^{-}	–	15^{-}	(1)
	–	–	–	23^{-}	(4)
	–	–	–	10^{-}	(2)
	$2.5^{-}–10$	10	–	–	(12)
	9^{-}	9^{-}	25^{+}	25^{+}	(This study)

[a] References: (1) Hagiwara et al. 1988b. Salinity, 10–30 g/l. Temperature, 15–30°C. (2) Hagiwara et al. 1988a. Temperature 10–30°C. (3) Hagiwara et al. 1989. Salinity, 8–32 g/l. (4) Hagiwara and Lee 1990. Temperature, 23–30°C. (5) Hino and Hirano 1984. Temperature, 15–30°C. (6) Hino and Hirano 1988. Salinity, 10–30 g/l. (7) Ito et al. 1981. Temperature, 10–40°C. (8) Lubzens et al. 1980. Salinity 3.8–38 g/l. (9) Lubzens et al. 1985. Salinity 2–48 g/l. (10) Miracle and Serra 1989. Salinity 9–24 g/l. Temperature 20–30°C. (11) Pascual and Yúfera 1983. Salinity 0–80 g/l. Temperature 15–43°C. (12) Pozuelo and Lubián 1993. Salinity 2.5–50 g/l. (13) Snell 1986. Salinity 5–40 g/l. Temperature 20–40°C

threshold for mictic reproduction (0.03 females/ml), and a continuous bisexual reproduction pattern – higher at higher densities – during its presence in the pond. In contrast, *B. rotundiformis* SS had a punctuated mictic pattern, with higher density threshold (0.09 females/ml) and showing a mixis peak in the late summer-early fall, just prior to the population crash. Our laboratory study supports the higher density threshold for mixis in the small *B. rotundiformis* SS species than in *B. plicatilis*. In spite of the important difference between absolute values in density thresholds for mixis in natural and laboratory populations, the qualitative relationships between density thresholds of our experimental strains are consistent with field data obtained by Carmona et al. (1995).

In the few laboratory studies in which *Brachionus* species is stated – or can be drawn – , *B. plicatilis s.s.* tends to decrease mixis when salinity and temperature increases in the ranges we have used here (see Table 4). This is in agreement with our results, as L1 strain displayed a strong mictic response to a decrease in salinity. This reaction is probably adaptive in their environment, protecting genotypes from very low salinity conditions, which are likely to occur during winter flooding in Torreblanca Marsh.

The tendency of *B. rotundiformis* to increase mixis with temperature and salinity (Table 4) is not confirmed by our results. *B. rotundiformis* SS exhibited a negative relationship between mixis and temperature, as also happened in our field study for *B. rotundiformis* (Carmona et al. 1995). The mictic response would anticipate the adverse low temperatures of winter, which are not suitable for population growth of these thermophilic strains. The larger *B. rotundiformis* species (SM) has been found in the marsh either in spring or in autumn, avoiding cold temperatures, and high salinity conditions. Surprisingly, the highest mictic ratios for SM2 strain were found at intermediate temperatures and salinities. It is likely that a narrower range of temperature and salinity would have been useful to detect adaptive responses in mictic ratio in this species.

We have found that specialization to different temperature and salinity conditions of the three species in conjuction with differences in their mictic patterns are expected to produce a 34% reduction in male-female heterogamic encounters. A higher reduction is likely in natural populations because of interspecific competition, which will reduce frequency of one or two species, and, consequently, number of heterogamic encounters in natural populations. Therefore, ecological specialization and bisexual patterns together with male mating preferences, which are highly but not completely homogamic, clearly differentiate these species. We can hypothesize that adaptation of populations to different environments involves divergence in temporal or spatial distribution, and it also promotes divergence in the timing of sex, first as a concomitant side-effect, in which the density dependence of sex plays a role, and second, as a probable result of divergent evolution in the cues of mixis, since environmental unsuitability is genotype-dependent.

The implications of divergence in sexual response to environmental cues for speciation could be important in other cyclical parthenogens. Thus, some work done in cladocerans, other important group of cyclical parthenogens (Hebert 1987), have found inter and intraspecific differences in the sexual response of clones from the *Daphnia longispina* complex to cues known to induce sexual reproduction in *Daphnia* (Spaak 1995). With a similar approach, Lynch (1983, 1985) proposed that differences in timing of sex could favor speciation in cyclical parthenogens.

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