Mixis in rotifers of the lineage 'Nevada', belonging to the *Brachionus plicatilis* species complex, under different feeding regimes

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Abstract

A strain of *Brachionus* 'Nevada', which belongs to the *Brachionus plicatilis* species complex, and is commonly found in European hatcheries, was investigated in terms of its mixis potential. Two feeding regimes used for mass culturing were employed. Rotifer populations were fed on phytoplankton (*Tetraselmis suecica*) and either baker's yeast, *Saccharomyces cerevisiae* (treatments A) or Culture Selco® (treatments B). In order to promote mixis, the salinity of the culture medium was reduced from 40 to 20 g L^{-1}. Indeed, the rotifer populations of lower salinity (A 20, B 20) showed a twofold increase in mixis rates compared with those of higher salinity (A 40, B 40). In addition, treatment A 20 showed significantly higher levels of mixis (22.59 ± 2.07%) compared with B 20 (16.56 ± 1.46%). The opposite trend was observed for the parthenogenetic growth rates (A 20: 0.46 ± 0.01; B 20: 0.62 ± 0.01). It is thus concluded that Culture Selco leads to a higher abundance of amictic ovigerous females, whereas yeast supports a higher abundance of males and mictic females carrying resting eggs. The two types of feeding regimes can be used for different purposes in a hatchery.

Keywords: rotifera, *Brachionus*, hatchery, mixis, population structure

Introduction

The reproduction of the monogonont rotifer, *Brachionus plicatilis*, comprises of alternating phases of parthenogenetic and sexual reproduction. Parthenogenesis occupies the largest part and is mediated through the propagation of parthenogenetic (amictic) females, which mitotically produce amictic eggs. Sexual (mictic) reproduction begins with mictic females, which meiotically produce haploid eggs that develop into males. These in turn fertilize young mictic females, and resting eggs are produced. Resting eggs mark the end point of the mictic phase. After a typically extended period of dormancy and upon receiving a hatching stimulus, the resting eggs will hatch into amictic females and the parthenogenetic cycle will start once again (Ruttner-Kolisko 1974).

The biological significance of mixis can be viewed in different contexts. On one hand, resting eggs represent the diapause stage of the reproductive cycle, a way to withstand unfavourable environmental conditions. Bearing in mind that rotifers occupy ephemeral habitats for fragmented periods of time, resting eggs appear to be the 'passport' of the population to the next temporal and/or spatial colonization. On the other hand, the genetic uniformity of parthenogenesis is interrupted by bouts of sexual recombination during mixis. In that way, the genetic pool is renewed with every new colonization event. An inevitable side effect of mixis is the dramatic reduction in the population potential for numerical increase. Resting eggs do not contribute to immediate population growth as they do not hatch right away (Pourriot & Snell 1983; Gilbert 1992).

Apart from the advantages of mixis, there are cases where parthenogenesis is the preferred mode of reproduction. Parthenogenesis results in rapid

population growth and colonization of a habitat. This mode of reproduction is required by aquaculture farms, where mass culturing of rotifers yields the first feed of fish larvae. Mixis and subsequent cyst formation are referred to as 'population crashes' and are unwanted events during the production cycle (Papakostas, Dooms, Triantafyllidis, Deloof, Kappas, Dierckens, De Wolf, Bossier, Vadstein, Kui, Sorgeloos & Abatzopoulos 2006).

The mechanism underlying the switch from parthenogenesis to mixis is therefore of both theoretical and practical interest. Several factors have been linked to the occurrence of mixis, such as temperature (Hino & Hirano 1984; Kogane, Hagiwara & Imaizumi 1997) and salinity decrease (Lubzens, Fishler & Bergudo-white 1980; Lubzens, Minkoff & Marom 1985; Hagiwara, Hino & Hirano 1988a), food quantity and quality (Ben-Amotz & Fishler 1982; Lubzens & Minkoff 1988; Snell & Boyer 1988). All these factors may have a modifying effect but do not induce mixis *per se* (Gilbert 1992). The signal shown to trigger mixis is high population density (Hino & Hirano 1976; Snell & Boyer 1988; Carmona, Serra & Miracle 1993; Carmona, Gómez & Serra 1995; Stelzer & Snell 2003). This connection stems from the widespread view that optimal conditions are required for mixis to occur. High population densities are a sign of a rapidly expanding population, which grows under favourable conditions that can support the energy-demanding production of resting eggs. Peak densities also increase the likelihood of encounters between male individuals and young mictic females, thus increasing the possibility of fertilization and subsequent resting egg formation (Gerritsen 1980; Pourriot & Snell 1983; Lubzens & Minkoff 1988; Serra, Snell & King 2004). The mode of action of the density effect has been attributed to the accumulation of a substance, quite possibly a protein, which is released into the medium by the rotifers themselves (Hino & Hirano 1976; Carmona *et al.* 1993; Stelzer & Snell 2003; Snell, Kubanek, Carter, Payne, Kim, Hicks & Stelzer 2006).

Nevertheless, variation in mixis has been observed in the response of different rotifer strains to identical sets of environmental conditions (Hino & Hirano 1977; Snell & Hoff 1985; Hagiwara, Hino & Hirano 1988b; Hagiwara & Lee 1991; Pozuelo & Lubián 1993; Carmona, Serra & Miracle 1994; Carmona *et al.* 1995). So far, this has been attributed to inter-strain variability. To further complicate matters, variation within the same strain (intra-strain) cultured under different conditions appears to be, in some cases, of the

same magnitude (Hino & Hirano 1976; Hagiwara *et al.* 1988a; Hagiwara & Lee 1991; Kogane *et al.* 1997). This great variability can be partly attributed to the poorly defined species status of *B. plicatilis*. A recent study has revealed that *B. plicatilis* is a species complex, comprising of three species and six lineages possibly representing different species (Gómez, Serra, Carvalho & Lunt 2002). The re-definition of the species status of the complex should therefore be incorporated in the study of mixis induction, in order to assess the universality of the mixis cues and possible deviations from the general pattern. Apart from the theoretical interest, this knowledge would prove useful in aquaculture practices, because strains with well defined characteristics can serve different purposes (Hino & Hirano 1977; Kotani & Hagiwara 2003). For example, strains with low or no mixis would be preferable for mass culture production. On the other hand, strains with high mixis investment could be used in the mass production of resting eggs; hatching of these eggs could be induced under controlled conditions and used in the same manner as *Artemia* cysts (Lubzens 1981; Hagiwara *et al.* 1988a).

The present study deals with one of the newly described lineages of the *B. plicatilis* complex, found in hatcheries around Europe (Papakostas *et al.* 2006). The experimental conditions were designed according to commonly applied mass culture practices (Kostopoulou, Miliou, Katis & Verriopoulos 2006). Two rotifer populations cultured under different feeding regimes were exposed to conditions promoting mixis (i.e. salinity decrease) and monitored throughout a 9-day batch culture.

Materials and methods

Experimental stock population

The rotifer strain, isolate K, with lorica length of ovigerous female (mean $\pm$ SE = 238.5 $\pm$ 0.6 μm; $n =$ 385) used in the present study was identified on the basis of mitochondrial gene COI sequencing and was named according to the nomenclature suggested by Gómez *et al.* (2002). It belongs to *Brachionus* 'Nevada' (GenBank accession number AM180752), a newly described lineage of the *B. plicatilis* species complex. *Brachionus* 'Nevada' has been grouped in the *B. plicatilis s.s.* clade (Gómez *et al.* 2002). Its presence has been confirmed in several hatcheries around Europe (Papakostas *et al.* 2006).

A pre-experimental low-density culture of a parthenogenetic population of *B.* 'Nevada' was

Table 1 Management of experimental rotifer cultures

| Treatment | Culture conditions | | | Culture volume (mL) | | | Rotifer initial quantity (day 0) | |
	T (°C)	S (g L^{-1})	Food type	Initial	Final	Daily addition	Density (ind. mL^{-1})	Total quantity (ind. 10^3)
A 40	25	40	1	50	450	50	200	10
A 20	25	20	1	50	450	50	200	10
B 40	25	40	2	170	330	20	60	10
B 20	25	20	2	170	330	20	60	10

Food type 1: baker's yeast (*Saccharomyces cerevisiae*) and phytoplankton (*Tetraselmis suecica*).
Food type 2: culture Selco and phytoplankton (*T. suecica*).

maintained under $T = 25$ °C, $S = 40$ g L^{-1}, constant illumination and aeration (Moretti, Pedini Fernandez-Criado, Cittolin & Guidastri 1999), using *Tetraselmis suecica* (LB 2286) as food. *T. suecica* was cultured in the medium described by Walne (1966) and modified by Laing (1991). The culture medium consisted of filtered (Whatman GF/C filter) and autoclaved (121 °C, 10 min) seawater. Where applicable, dilution was carried out with double distilled water. Stock and experimental populations were maintained in a temperature-controlled chamber.

Experimental procedure

The experiment was conducted in 500 mL round-bottom Pyrex glass conical flasks. Four treatments were performed A 40, A 20, B 40 and B 20, with three replicates each. The first component (letter A or B) denotes the feeding regime and the second (number 40 or 20) represents the salinity of the medium (Table 1). Rotifers were batch cultured for 9 days and 1 mL samples were collected twice daily, one at 08:00 hours and another at 16:00 hours for quantitative and qualitative analysis of the population. The rotifer samples were preserved in 4% buffered formalin.

The feeding regimes used were typical of Mediterranean hatcheries. Food consisted of either baker's yeast *Saccharomyces cerevisiae* and phytoplankton *T. suecica* (treatments A) or Culture Selco® (INVE N.V., Gent, Belgium) and phytoplankton *T. suecica* (treatments B – Table 1). Culture Selco is a nutritionally boosted, lipid-rich artificial feed, particularly enriched in highly unsaturated fatty acids (HUFAs) (Dhert, Rombaut, Suantika & Sorgeloos 2001). Its dry weight composition consists approximately of 15% lipids, 35% proteins and 30% carbohydrates (Suantika, Dhert, Nurhudah & Sorgeloos 2000), yielding a ratio protein to lipid of 2.3. Baker's yeast, on the other hand, is not as nutritionally boosted, with a composition of 8% lipids, 30% proteins and

46% carbohydrates (Frolov, Pankov, Geradze, Pankova & Spektorova 1991), yielding a ratio protein to lipid of 3.75. The quantity of phytoplankton supplied daily to the rotifer cultures was different in the two feeding regimes: in the case of yeast, phytoplankton was added in order to improve the nutritional value of the diet (Hirayama & Watanabe 1973), whereas in the case of Culture Selco, a lesser amount of phytoplankton was required, so as to improve the quality of the culture medium (Dhert *et al.* 2001). According to the above, phytoplankton was close to (1.5×10^4 cells mL^{-1} in treatments A) or less than (0.2×10^4 cells mL^{-1} in treatments B) 'green water' levels (Reitan, Rainuzzo, Øie & Olsen 1993; Dhert *et al.* 2001) and, in either case, not high enough to satiate rotifers (Hirayama & Ogawa 1972). In addition, a 20-fold increase in the levels of phytoplankton supplied daily did not yield differences in the dynamics of the rotifer populations of the two feeding regimes (Kostopoulou *et al.* 2006). As a result, phytoplankton was not considered to play a significant role in the determination of the dynamics of the studied rotifer populations. The amounts of yeast and Culture Selco corresponded to recommended levels for mass culture production (Moretti *et al.* 1999; Suantika *et al.* 2000) and were therefore regarded as satiating. The water quality of the culture medium was similar in all treatments (mean ± SE: pH = 7.41 ± 0.03, NH_4^+ = 0.48 ± 0.09 mg L^{-1}, NH_3 = 0.50 ± 0.09 × 10^{-2} mg L^{-1}, NO_3^- = 4.55 ± 0.40 mg L^{-1}, $NO_2^=$ = 0.26 ± 0.06 mg L^{-1}).

All treatments were initiated with arithmetically equal rotifer populations (10^4 individuals (ind.) – Table 1). A different handling protocol was followed for each feeding regime, as a result of the different phytoplankton quantity added daily. The starting volume was set to be lower in treatments A (50 mL), compared with B (170 mL), in order to allow for the greater volume (phytoplankton) addition, required in the former treatments. Specifically, the daily addition of

medium in the experimental flasks was 50 and 20 mL in treatment A and B respectively. As a consequence of the different starting volumes, the initial rotifer densities were inevitably higher in treatments A (200 ind. mL^{-1}) than in B (60 ind. mL^{-1}). Nevertheless, previous experiments showed that these differences in initial rotifer densities were smoothed out from day 2 onwards in a 4-day rotifer batch culture (Kostopoulou *et al.* 2006). Each of the two feeding regimes was applied under two salinities (40 and 20 g L^{-1}), in order to monitor the expression of mixis when salinity is decreased from mass culture practices (40 g L^{-1}) to more favourable conditions (20 g L^{-1}).

Data analysis

The preserved rotifer samples were inspected under an inverted microscope (ZEISS, Carl Zeiss, Jena, Germany). The qualitative analysis of the population included the following phases. The parthenogenetic life cycle was divided into the following: immature individuals, non-ovigerous females, females with single egg,

The quantitative analysis of the population included the following indices. The population growth rate (r, ind. day^{-1}) was calculated as the slope of the semi-log plot of rotifer total numbers versus time during the exponential growth phase:

$$N_t = N_o \, e^{rt}$$

where $N_{o,t}$ is the rotifer total quantity (density × volume) at 24-h intervals and r is the growth rate, which is calculated as the slope of the regression line. The intercept corresponds to the initial total quantity of rotifers.

Three different growth rates were determined (Table 2): for the amictic growth rate, total rotifer numbers excluding the males were used. The male-producing mictic female growth rate and male growth rate were estimated using the total numbers of the respective life cycle phases.

Sexual reproduction was quantified by means of the mixis and fertilization rates:

$$\text{Mixis rate} = \frac{\text{total quantity of mictic females (male- and resting egg-producing)}}{\text{total quantity of ovigerous amictic and mictic females}}$$

$$\text{Fertilization rate} = \frac{\text{total quantity of resting egg- producing mictic females}}{\text{total quantity of mictic females (male- and resting egg- producing)}}$$

females with multiple eggs, females with sac (i.e. external membrane of the egg after hatching) and post-reproductive females. The latter have been described by Carmona, Serra and Miracle (1989) and Ricci and Fascio (1995) as having degenerate digestive organs and gonads and general swelling of the body. This categorization of the rotifer life cycle has been used to describe population dynamics under mass culture conditions promoting parthenogenesis (Kostopoulou *et al.* 2006). The mictic life cycle included the male-producing mictic females, males and resting egg-producing mictic females. The ovigerous females were differentiated on the basis of their eggs; male-producing mictic eggs are smaller than amictic and resting eggs. The former eggs are usually carried in large numbers. Resting eggs are the largest in size, have thick walls and dark colouring. Amictic eggs have intermediate size (Ruttner-Kolisko 1974). The abundance (%) of each life cycle phase was estimated as its density divided by the total density and multiplied by 100.

In addition, the male:female ratio was estimated from the total number of males and male-producing mictic females.

The data collected during the experiments was subjected to statistical analysis. Normal distribution and homogeneity of variance were assessed before analysis and the data was accordingly transformed. One-way analysis of variance (ANOVA) was performed to determine any significant differences ($P < 0.05$) between the slopes and intercepts of the regression lines determining the aforementioned growth rates. Analysis of co-variance (ANCOVA) (using time as covariate) was used to test significance of differences between the experimental treatments in the various calculated parameters. In particular, two-way ANCOVA (using time as covariate) was applied to the population structure data, in order to identify main effects and interaction of factors; feeding regime (A, B) and salinity (20, 40 g L^{-1}). Comparison of means was conducted with LSD multiple range test.

Table 2 Measured population indices (mean ± SE) in the rotifer *Brachionus* 'Nevada'

| Treatment | Population growth rate (ind. day^{-1}) | | | Mixis rate (%) | Fertilization rate (%) | Male:female |
	Amictic	Male-producing mictic female	Male			
A 40	0.31 ± 0.02^a	0.26 ± 0.04^a	0.40 ± 0.04^a	8.79 ± 0.55^a	–	0.61 ± 0.11^a
A 20	0.46 ± 0.01^b	0.62 ± 0.05^b	0.76 ± 0.06^c	22.59 ± 2.07^c	15.86 ± 4.53^b	2.35 ± 0.31^c
B 40	0.56 ± 0.02^c	0.28 ± 0.01^a	0.25 ± 0.09^a	10.81 ± 0.86^a	–	0.42 ± 0.06^a
B 20	0.62 ± 0.01^d	0.63 ± 0.09^b	0.55 ± 0.05^b	16.56 ± 1.46^b	1.68 ± 0.71^a	1.37 ± 0.14^b
P	***	**	***	***	*	***

Means within a column having a different letter in superscript are significantly different.

*$P < 0.05$;

**$P < 0.01$;

***$P < 0.001$; $n = 3$.

Results

With reference to the mictic life cycle phases, ANCOVA was applied from the day of first appearance onwards (Statgraphics, Statistical Graphics Corp., Herndon, VA, USA). Untransformed mean values ± standard error (SE) are presented. The measurements taken at the beginning of the batch cultures (day 0) were not included in the statistical tests.

Population growth rate (Table 2) was significantly higher for amictic ($P < 0.001$), male-producing mictic females ($P < 0.01$) and males ($P < 0.001$) in the lower salinity treatments (A 20, B 20) compared with the higher salinity ones (A 40, B 40). In regards to the feeding regimes, the amictic growth rate was higher in treatments B (Culture Selco-fed) compared with A (yeast-fed). The male growth rate, mixis rate and male:female ratio were significantly higher ($P < 0.001$) in A 20, followed by B 20 and lower in A 40 and B 40. Between the latter two, no significant difference was observed. No resting egg-producing mictic females or resting eggs were detected in the higher salinity treatments (A 40, B 40), hence fertilization rate was not calculated. At the lower salinity ($20\,g\,L^{-1}$), the fertilization rate was higher ($P < 0.05$) in A compared with B.

Regarding the temporal variation in total numbers of rotifers (Fig. 1a), male-producing mictic females (Fig. 1b) and males (Fig. 1c) among treatments, differences became evident after the fifth day of culture, by which time B 20 broke off from the rest of the treatments and reached the highest levels. The next-to-highest values were observed for males in A 20, whereas values of male-producing mictic females were similar in A 20 and B 40. The latter appeared elevated in B 20 from day 3 onwards, reaching the

highest levels after day 5, when males were also highest. The lowest numbers were recorded in treatment A 40. The mixis rate differentiated between the low and high salinity treatments from day 2 onwards, with the exception of day 7 at 8:00 hours (Fig. 2a). Between A 20 and B 20, differences in mixis rate were observed from the fourth day onwards, except from day 6 and day 8 at 8:00 hours.

Male-producing mictic females first appeared in the low salinity treatments on day 2, and males on day 3 (Table 3). No differences were observed in their initial densities, whereas B 20 was characterized by significantly higher initial total quantities, compared with A 20. In accordance with this finding, the intercepts of the calculated growth rates of the respective phases were also significantly different, showing that the first-appearing total numbers were higher in B 20 than in A 20. In contrast, the intercepts of the amictic population growth rates did not differ, verifying the similar initial total quantities that were used to start the experiments. For the mean and final estimates of male-producing mictic females and males, the trend shown by density coincided with that of the total quantity of individuals of the respective phases (except for final estimates of males). Low salinity treatments (A 20, B 20) showed significantly higher values than the corresponding high salinity treatments (A 40 and B 40 respectively) and B 20 was significantly higher than A 20 (except for final total quantity of males, where they were similar). The resting egg-producing mictic females did not show any significant differences between A 20 and B 20 for either density (mean, A 20: 1.00 ± 0.32; B 20: 0.60 ± 0.25) or total quantity (mean, A 20: 410 ± 140; B 20: 190 ± 80). With respect to the high salinity treatments, B 40 was similar to A 40 for final estimates of males and male-producing mictic
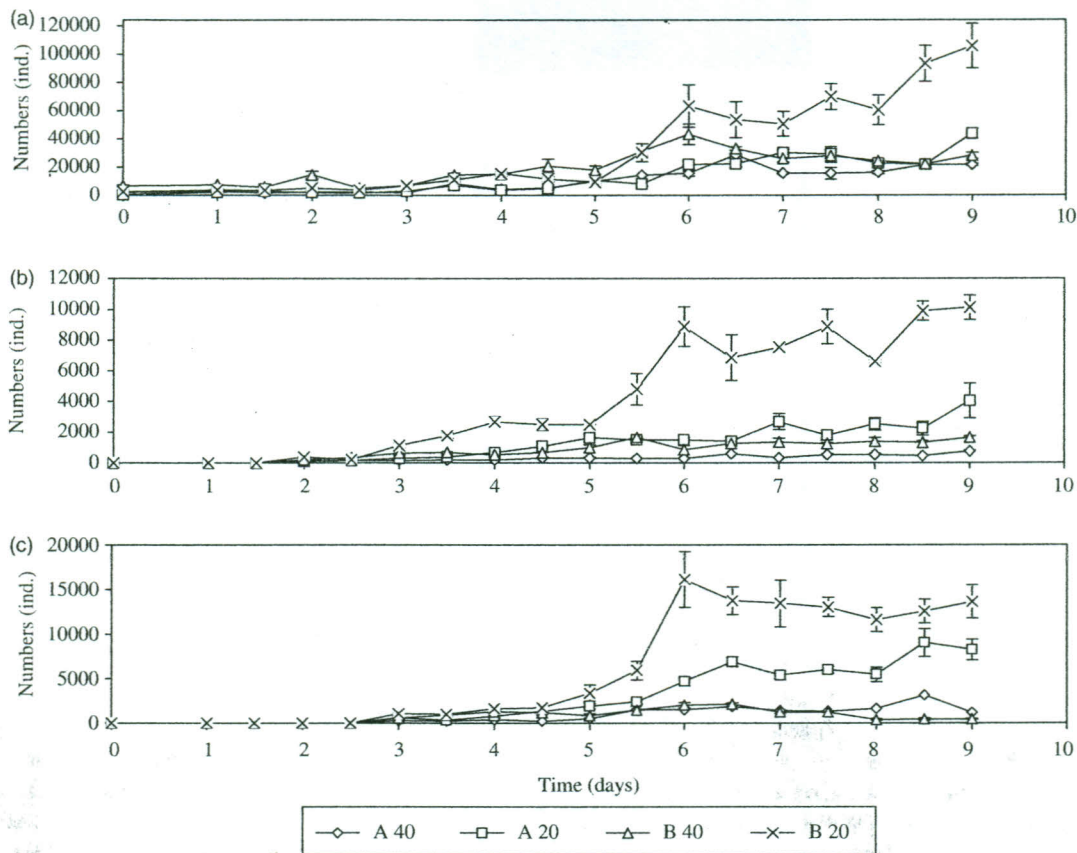
Figure 1 Temporal variation in total numbers (mean ± SE) of (a) rotifers, (b) male-producing mictic females and (c) males during the 9-day culture of *Brachionus* 'Nevada' in the four experimental treatments (A 40, A 20, B 40, B 20; see Table 1).

females, higher for the mean estimates of the latter and lower for the mean estimates of males.

Initial rotifer density (day 0) was set to be higher in treatments A than in B (Table 1). However, this trend was reversed during the course of the experiment (Fig. 2b). From day 1 onwards, no significant differences were found among treatments, except for the last 2 days, where B 20 exceeded the rest of the treatments. Overall, treatment B 20 had the highest mean rotifer density ($P < 0.001$), followed by B 40, A 20 and A 40 in decreasing order (Table 3). Final rotifer density was maximum ($P < 0.01$) in B 20, while values for the other treatments were similar. Mean rotifer total quantity was significantly higher in treatments B compared with A ($P < 0.01$), whereas final total quantity was higher in B 20 ($P < 0.01$) and similar in the rest of the treatments.

The qualitative analysis of the population in the four treatments is shown in Table 4. Low salinity treatments (A 20, B 20) were characterized by significantly higher abundance (%) of mictic life cycle phases and females with single egg and lower non-ovigerous females and immature individuals, compared with the higher salinity treatments (A 40, B 40). Between the lower salinity treatments, B 20 had an increased abundance of amictic ovigerous females than A 20. In contrast, A 20 had significantly higher abundance of amictic non-ovigerous females, males and resting egg-producing mictic females, compared with B 20. The abundance (%) of male-producing mictic females did not differ between A 20 and B 20 for the whole experimental period (Fig. 2c). Between the higher salinity treatments, B 40 had an increased abundance of immature individuals and amictic females with multiple eggs and lower levels of non-ovigerous females, compared with A 40. Finally, Culture Selco-fed rotifer populations (treatments B) had a significantly higher abundance of
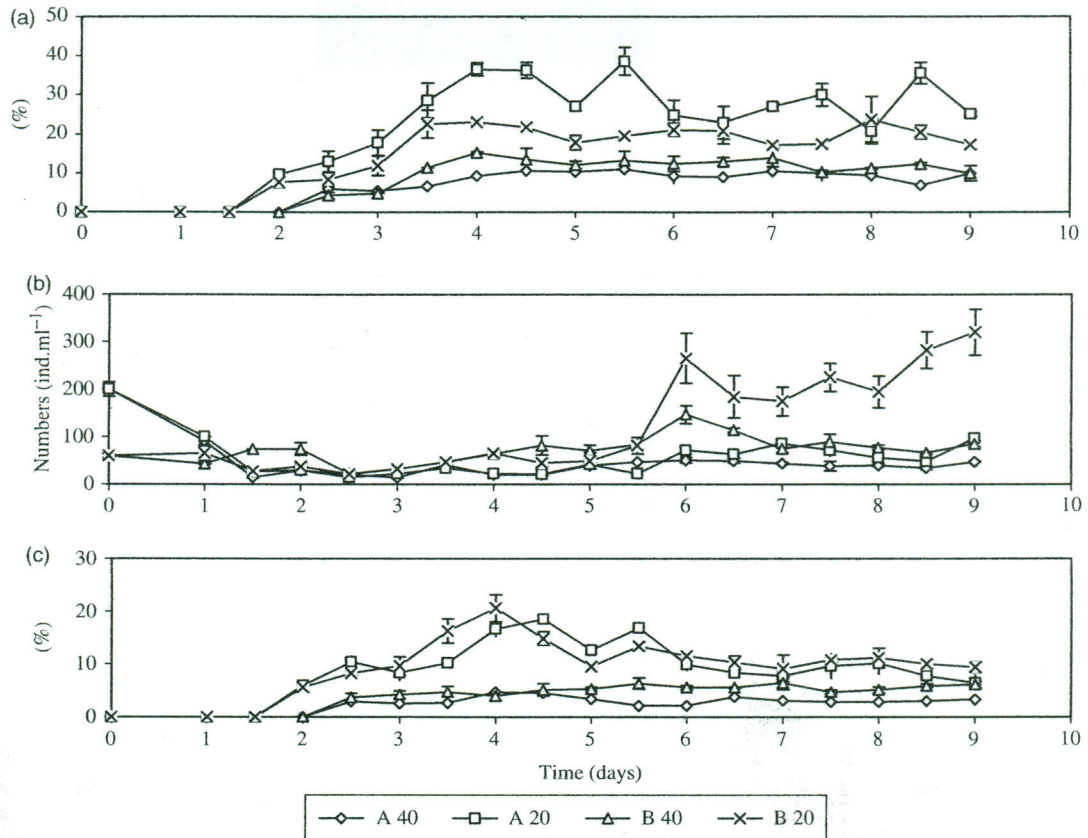
Figure 2 Temporal variation of rotifers (mean $\pm$ SE) in terms of (a) mixis rate (%), (b) density (individuals mL^{-1}) and (c) abundance (%) of male-producing mictic females during the 9-day culture of *Brachionus* 'Nevada' in the four experimental treatments (A 40, A 20, B 40, B 20; see Table 1).

females with multiple eggs compared with yeast-fed populations (treatments A), irrespectively of salinity. On the other hand, male-producing mictic females and males were influenced by both factors (i.e. feeding regime and salinity), which acted independently of each other.

Discussion

The present study examines the response of *Brachionus* 'Nevada' populations, reared under two different feeding regimes, when salinity is lowered from 40 to 20 g L^{-1}. In the higher salinity treatments, the feeding regime influenced the rotifer population dynamics quantitatively as well as qualitatively. The feeding regime of treatment A, comprising mainly of yeast, yielded rotifer populations of lower amictic growth rates, compared with those of treatment B

(Culture Selco). The former populations consisted of non-ovigerous females and the amictic ovigerous females mainly carried single eggs. The latter populations were characterized by increased abundance of immature individuals and amictic females carrying multiple eggs. These differences in amictic growth rates (Table 2) and population structure (Table 4) are in accordance with a previous study (Kostopoulou *et al.* 2006) using the same feeding regimes.

In the lower salinity treatments (A 20, B 20), the amictic growth rate as well as mixis was substantially increased in both feeding regimes compared with the higher salinity ones, as also shown in previous studies (Lubzens *et al.* 1980, 1985; Snell & Hoff 1985; Pozuelo & Lubián 1993). The mechanism of mixis modulation via salinity decrease lies in the higher amount of energy that is available in the lower salinity conditions. Under such conditions, filtration rates increase (Hirayama & Ogawa 1972) and thus

Table 3 Average values (SE) of density (ind. mL^{-1}) and total quantity (ind.) of rotifers, male-producing mictic females and males on the first day of appearance (first appearance), during the whole sampling period (mean) and on the final day of culture (final). For treatments see Table 1

	Rotifers				Male-producing mictic females						Males					
	Mean		Final		First appearance†		Mean		Final		First appearance‡		Mean		Final	
Treatment	Density (ind. mL^{-1})	Total (ind.10³)	Density (ind. mL^{-1})	Total (ind.10³)	Density (ind. mL^{-1})	Total (ind.10³)	Density (ind. mL^{-1})	Total (ind.10³)	Density (ind. mL^{-1})	Total (ind.10³)	Density (ind. mL^{-1})	Total (ind.10³)	Density (ind. mL^{-1})	Total (ind.10³)	Density (ind. mL^{-1})	Total (ind.10³)
A 40	34.43[a] (2.45)	9.90[a] (1.22)	47.67[a] (3.48)	21.45[a] (1.57)	—	—	1.20[a] (0.08)	0.30[a] (0.03)	1.67[a] (0.33)	0.75[a] (0.15)	—	—	3.21[b] (0.42)	1.02[b] (0.15)	2.67[a] (0.88)	1.20[a] (0.40)
A 20	49.15[b] (4.56)	11.98[b] (1.81)	97.00[a] (16.26)	43.65[a] (7.32)	1.00 (0.00)	0.05[a] (0.00)	3.49[b] (0.42)	1.00[b] (0.14)	9.00[b] (5.00)	4.05[b] (2.25)	2.00 (0.58)	0.22[a] (0.04)	8.17[c] (1.07)	2.76[c] (0.44)	18.33[b] (5.24)	8.25[b] (2.36)
B 40	72.47[c] (5.65)	19.51[c] (2.02)	85.00[a] (13.28)	28.05[a] (4.38)	—	—	3.28[b] (0.29)	0.89[b] (0.09)	5.00[ab] (0.58)	1.65[ab] (0.19)	—	—	3.00[a] (0.42)	0.88[a] (0.13)	1.33[a] (0.33)	0.44[a] (0.11)
B 20	136.78[d] (19.31)	26.88[d] (4.41)	320.67[b] (96.53)	105.82[b] (31.85)	1.33 (0.33)	0.23[b] (0.06)	13.36[c] (1.77)	4.07[c] (0.58)	30.67[c] (4.81)	10.12[c] (1.59)	3.00 (0.00)	0.57[b] (0.00)	20.00[d] (3.18)	5.68[d] (0.91)	41.33[c] (11.21)	13.64[b] (3.70)
P	***	**	**	**	NS	*	***	***	***	**	NS	**	***	***	**	**

Means within a column having a different letter in superscript are significantly different.

*$P < 0.05$;

**$P < 0.01$;

***$P < 0.001$; NS, non significant; $n = 3$.

†Day 2.

‡Day 3.

Table 4 Abundance (%) of life cycle phases according to treatment (A 40, A 20, B 40, B 20; see Table 1)

	A 40	A 20	B 40	B 20	P_{food}	P_{sal}	$P_{foodxsal}$
Immature	7.61 ± 0.92[b]	4.07 ± 0.62[a]	15.78 ± 1.29[c]	4.09 ± 0.91[a]	***	***	***
Non-ovigerous females	51.40 ± 1.58[c]	29.29 ± 1.99[b]	30.00 ± 1.85[b]	14.14 ± 1.00[a]	***	***	NS
Females with single egg	21.04 ± 0.72[a]	27.39 ± 1.74[b]	19.65 ± 0.89[a]	31.53 ± 1.59[c]	NS	***	*
Female with multiple eggs	7.73 ± 1.06[a]	8.92 ± 1.29[a]	21.50 ± 1.25[b]	24.15 ± 2.21[b]	***	NS	NS
Female with sac	1.30 ± 0.36	2.06 ± 0.41	1.64 ± 0.27	0.86 ± 0.17	NS	NS	***
Post reproductive female	1.06 ± 0.45	1.33 ± 0.31	2.12 ± 0.41	0.88 ± 0.16	*	NS	**
Male-producing mictic female	3.47 ± 0.30[a]	9.20 ± 0.83[b]	5.04 ± 0.41[a]	10.61 ± 0.81[b]	**	***	NS
Male	6.39 ± 0.69[a]	17.39 ± 1.61[c]	4.27 ± 0.48[a]	13.72 ± 1.35[b]	**	***	NS
Resting egg-producing mictic female	–	0.35 ± 0.19[b]	–	0.02 ± 0.01[a]	–	*	–

Values appear as mean ± S.E.

Means within a row having a different letter in superscript are significantly different.

P_{food} for feeding regime effects (A, B); P_{sal} for salinity effects (20, 40 g L^{-1}); $P_{foodsal}$ for interaction of both factors (two-way ANCOVA).

*$P < 0.05$;

**$P < 0.01$;

***$P < 0.001$; NS, non significant; $n = 3$.

higher amounts of food are consumed. In addition, higher amounts of energy are allocated to reproduction, due to the lower energy requirements of osmoregulation (Lubzens *et al.* 1985). Thus, when excess energy becomes available, the conditions arise for a, partial at least, switch to mixis that would ultimately result in the production of energy-rich resting eggs (Gilbert 2004).

The first appearance of male-producing mictic females was recorded on day 2 and was closely followed by the appearance of males. Resting egg-producing mictic females were recorded towards the end of the experiment (day 7). This temporal pattern of appearance has been reported in previous studies, irrespective of the set of conditions chosen as a trigger/modulator for mixis (Lubzens *et al.* 1980; Ben-Amotz & Fishler 1982; Snell & Hoff 1987). The levels of mixis recorded in the present study for B. 'Nevada' fluctuated around 20%, revealing a partial switch to mixis. This bet-hedging strategy ensures that some (parthenogenetic) population growth still occurs, while resting eggs are produced to secure survival in case of future habitat deterioration (Snell 1987).

In a compilation of studies using small- or large-scale cultures of the *B. plicatilis* species complex (Table 5), it can be seen that mixis rates show as much inter- (column 3) as intra- (column 4) strain variability. Although this table makes use of crude taxonomic criteria (large/small morphotypes) and different experimental conditions, some suggestions can still be made. The average of all reported mixis rates is 24.6 ± 4.5%, a value close to our estimates corresponding to the low salinity treatments. Similarly, all strains show a partial switch to mixis. It is not possible to draw a pattern with regard to species/morphotype. This could be attributed to either environmental and/or genetic variability and future studies should be conducted taking into consideration the new taxonomic scheme.

Differences in amictic growth rate and mixis were also observed between the populations of the two feeding regimes under low salinity conditions. Mixis was higher in yeast-fed rotifers (treatment A 20: 22.59 ± 2.07%), compared with the ones fed on Culture Selco (treatment B 20: 16.56 ± 1.46%). The parthenogenetic growth rate followed the opposite pattern from the mixis rate. This finding contrasts with the positive relationship between the mixis rate and the (amictic) growth rate reported in other studies (Lubzens *et al.* 1985; Snell 1987; Snell & Boyer 1988; Pozuelo & Lubián 1993), a relationship that reflects the connection between occurrence of mixis and optimal conditions. The fertilization rate produced similar results to mixis levels, being higher in yeast-fed rotifers (A 20). The factors responsible for the observed difference in mixis rates between the two treatments could be either exogenous or endogenous; exogenous factors would be those arising from the experimental environment, i.e. rotifer density, volume increase and/or food type. Endogenous factors would be the response of the rotifers to this environment, i.e. the rate of production of the different mictic stages, fecundity and mortality.

Changes in rotifer density or culture medium result in the chemical modification of the medium, which has been shown to affect mixis. It has been argued that metabolites released by the rotifers themselves trigger mixis, and volume addition dilutes

Table 5 Mixis rate (%) reported in the literature for the *Brachionus plicatilis*-species complex

Species	Strain	Mixis rate (%)	Range	Reference
B. rotundiformis	Hamana S-type	0.7		Hagiwara *et al.* (1988b)
B. rotundiformis	Mie S-type	59.1		Hagiwara *et al.* (1988b)
B. rotundiformis	Manado	25.18 ± 2.22	22.96–27.40	Rumengan *et al.* (1998)
B. rotundiformis	Langkawi SS-type	24.1 ± 6.37	13.2–37.5	Assavaaree *et al.* (2003)
B. plicatilis	Hawaiian S-type	46.22 ± 18.68	8.7–81.0	Hagiwara & Lee (1991)
B. plicatilis	O186S-type	3.78 ± 0.63	2.5–6.1	Hamada *et al.* (1993)
B. plicatilis		20.66 ± 7.30	0–62.5	Hino & Hirano (1976)
B. plicatilis	Tokyo L-type	22.87 ± 5.80	1.9–47.3	Hagiwara *et al.* (1988a)
B. plicatilis	Tokyo L-type	39		Hagiwara *et al.* (1988b)
B. plicatilis	Mie L-type	18.1		Hagiwara *et al.* (1988b)
B. plicatilis	Yashima L-type	0.3		Hagiwara *et al.* (1988b)
B. plicatilis	Tokyo L-type	20.5 ± 3.89	5.3–27.9	Hagiwara & Lee (1991)
B. plicatilis	NH1L-type	17.70 ± 2.88	9.8–25.7	Hamada *et al.* (1993)
B. plicatilis	Kamiura	20.46 ± 5.87	0–59.6	Kogane *et al.* (1997)
B. plicatilis	L1	50.75 ± 9.17	30–69	Aparici *et al.* (2002)

Values appear as mean ± SE.

their effect, thus decreasing mixis (Hino & Hirano 1976; Carmona *et al.* 1993; Stelzer & Snell 2003; Snell *et al.* 2006). Taking into consideration that the mixis signal acts on the developing oocyte (Snell *et al.* 2006), we should expect to observe mixis in both treatments (A 20, B 20) 2 days after the initiation of the experiment, which corresponds to the time needed for the first egg to be extruded in our strain at $T = 25$ °C (unpublished data). Indeed, male-producing mictic females first appeared on day 2 in both treatments. However, no significant differences in mixis rates were found on days 2 and 3 between treatments A 20 and B 20. This means that the differences in initial rotifer density and volume of the two treatments did not affect the mixis rate, probably because they were well above threshold levels triggering mixis (one rotifer per $15 \, \text{mL}^{-1}$ – Snell *et al.* 2006). Furthermore, in treatment B 20 the dilution was of smaller magnitude compared with A 20 and rotifer densities were similar between days 1 and 6 and higher in B 20 thereafter. However, the mixis rate was higher in A 20 from day 4 onwards. Therefore, rotifer density and/or volume changes cannot explain the variation in mixis rates obtained in the present study.

The type of diet – mainly in the form of different phytoplankton species – has also been shown to affect mixis rates (Hamada, Hagiwara & Hirayama 1993; Rumengan, Warouw & Hagiwara 1998). Adding baker's yeast to a phytoplankton-based diet has a positive effect on mixis (Snell & Hoff 1985). In the present study, the addition of yeast also had a positive influence on mixis in *B.* 'Nevada', in relation to the ad-

dition of a lipid-rich diet. To the best of our knowledge, the reason for the observed response has not been described yet.

In order to explore the endogenous mechanism yielding the different mictic rates, the characteristics of the rotifer populations of the low salinity treatments (A 20 and B 20) will be discussed. The mictic growth rate (mictic female *r* and male *r* – Table 2) differed between the feeding regimes only in the case of males. The levels of male *r* fell within previously reported values (Snell 1986; Pozuelo & Lubián 1993). This difference in male growth rates means that the rate of production of males was influenced by the type of diet, but that of male-producing mictic females was not. Besides, the abundance (%) of male-producing mictic females was similar between A 20 and B 20 for the whole experimental period. On the other hand, although the initial total quantity of males was higher in B 20 compared with A 20, the final one was similar in both treatments, probably due to the higher male *r* in A 20.

To reinforce this finding, differences were also observed in the male:female ratios. The populations of treatment A 20 showed a higher male:female ratio compared with those of treatment B 20 (also inferred from the qualitative analysis of the populations – Table 4). Consequently, more males were present in the populations of A 20, relative to the number of male-producing mictic females. According to Snell and Hoff (1985) and Snell (1986), the number of males in a population is determined by the growth rate of mictic females, the fecundity of each individual female and male survival. As the growth rate of male-produ-

cing mictic females was similar in the two treatments, the first determinant can be, to an extent, rejected. Male survival has been proposed as a possible explanation at environmental extremes (Snell 1986), which is not the case here. Our data therefore points towards differences in the fecundity of the above-mentioned females. It appears that yeast-fed male-producing mictic females produce more males than Culture Selco-fed ones.

It is suggested that the observed response stems from differences that characterize amictic, mictic females, and their subsequent offspring. In this study, lipid-rich Culture Selco positively influenced the fecundity of amictic females, whereas the food type with the higher protein to lipid ratio (yeast) influenced that of male-producing mictic females. Other factors, such as temperature, salinity, ammonia, food type and concentration have been shown to affect these types of females in a different way (Snell & Hoff 1985; Snell 1986; Snell & Boyer 1988). It has been argued that mictic females require optimal conditions and higher food concentrations than amictic ones and, quite possibly, special resources in order to be produced (Serra *et al.* 2004). This is to be expected since these types of females differ in their reproductive physiology (i.e. in their life histories, size and type of eggs they produce and lifetime fecundity) (Ruttner-Kolisko 1974; Hagiwara *et al.* 1988a; Dahril 1997; Xi, Huang & Jin 2001).

From this study, it is obvious that amictic growth rate estimates and densities as well as total quantities do not necessarily give an indication of mixis. The arithmetic superiority of the population with the higher amictic growth rate resulted in more male-producing mictic females being present in the population. However, this did not result in higher mixis rates, due to the higher number of amictic ovigerous females. In this case, it was the abundance (%) and the growth rate of males that provided a possible explanation for the observed response. The latter has been used as an index of mixis by Snell and Hoff (1985) and Snell (1986). To sum up, it can be concluded that the relative and not the absolute investment in mixis should be preferentially used to explain differences in mixis rates.

In the present study and under the tested conditions, it was demonstrated that the outcome of mixis must have been mainly dictated by the fecundity of male-producing mictic females. The food type influenced the fecundity of the above-mentioned females possibly through the different composition of the two tested diets. Culture Selco, which is a lipid-rich diet,

was linked to an increased abundance of amictic ovigerous females, whereas yeast, having a higher protein to lipid content, was linked to a higher abundance of males, which resulted in more resting egg-producing mictic females. The latter diet led to rotifer populations of lower parthenogenetic growth rate and higher mixis. On the other hand, the lipid-rich diet gave rise to rotifer populations of higher parthenogenetic growth rate and lower mixis, both of which are considered advantageous for mass culture production of rotifers. The first step should therefore be to choose the appropriate rotifer strain, and then to use the adequate feeding regime, tuned to the spatial and temporal needs of each hatchery.

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