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Crossed induction of sex in sympatric congeneric rotifer populations

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Abstract

The transition from asexual to sexual reproduction in rotifers of the genus *Brachionus* (Monogononta) is triggered by a chemical signal produced and released by the rotifers themselves; the signal accumulates in the environment as population density increases. The response to this signal has been hypothesized to be species-specific. If not, the timing of sex and final diapausing egg production of a species might not be optimized. In this study, a set of experiments—based on individual and mass culture approaches—was carried out to investigate the differentiation in sex induction signals between sympatric and allopatric congeneric *Brachionus* populations by analyzing the capability of the medium conditioned by the growth of a population (i.e., medium containing the inducing signal) to induce sex in a different one. We found that populations induce sex in response to medium conditioned by congeneric populations. Results suggest differences among species in their production and responsiveness to sex-inducing signals, as well as slight differentiation in these signals. The observed lack of strong differentiation between sympatric populations is proposed to have implications in avoidance of competition.

Fitness consequences of every action and behavior in an animal (e.g., feeding, defense against predators, mating, and space or time dispersal) depend on the reception and correct interpretation of information coming from its environment. One particularly interesting example of this dependence is the timing of sex in cyclically parthenogenetic organisms. Cyclical parthenogens reproduce mostly asexually but also show bouts of sexual reproduction. This life cycle, thought to combine the advantages of recombination and the advantage of avoiding the cost of males in most generations, has evolved independently several times. Although only present in ~15,000 animal species (Hebert 1987), this cycle is common in the continental plankton in that cladocerans and monogonont rotifers are cyclical parthenogens. Not surprisingly, cyclical parthenogenesis is seen as an adaptation to time-varying environments (Bell 1982). In cyclical parthenogens, the transition from asexual to sexual reproduction, which has a large effect on population fitness (Serra et al. 2004; *see* also below), is triggered by an environmental signal that seems to inform the animals reliably of the appropriate moment for the switch to occur (McLinn and Stephens 2006).

In their typical life cycle, monogonont rotifer populations annually colonize the water column after diapausing eggs hatch from the sediments (Gilbert 1974; Pourriot and Snell 1983). After an initial phase that includes several generations of exclusively asexual (parthenogenetic) growth that allows the rapid colonization of the habitat, sexual reproduction (also called mixis) is induced by environmental factors such as population density (Snell and Boyer 1988; Carmona et al. 1993, 1994), dietary conditions (Gilbert 1980), or photoperiod (Pourriot 1963; Pourriot and Clément 1975), although asexual reproduction does not stop completely. It is important to note that sexual females are produced asexually, their sexual condition being determined during their own embryonic development (Snell and Childress 1987; Gilbert 2007). These sexual females produce haploid eggs that develop into diapausing eggs or males, depending on whether they are fertilized or not. Diapausing eggs will be produced only if males are previously present in the population to fertilize those haploid eggs. Diapausing eggs, which accumulate and remain viable for decades in sediments, allow the populations to survive during adverse conditions in the water column (García-Roger et al. 2006). Because sex is the way to produce diapausing eggs (i.e., the among-year measure of biological fitness) and thus to survive adverse periods in the water column (Serra and King 1999; Simon et al. 2003), it is crucial for these organisms to be coordinated for sexual reproduction.

The transition from asexual to sexual reproduction in rotifers of the genus *Brachionus* (Monogononta) is triggered by a chemical signal that is produced and released by the rotifers themselves and that accumulates in the environment as population density increases (Gilbert 2003; Stelzer and Snell 2003, 2006). A recent study by Snell et al. (2006) has shown that the chemical signal inducing sexual reproduction in *Brachionus plicatilis* is a water-soluble protein called mixis-inducing protein (MIP). Recently, Kubanek and Snell (2008) have proposed that the induction of sexual reproduction in monogonont rotifers seems to be the first described example of quorum sensing among aquatic animals, a mechanism whereby the accumulation of signaling molecules enables a single individual to sense and respond to the total number of individuals (i.e., population density).

A hypothesis can be proposed that the response to MIP (i.e., sexual reproduction induction) is species specific. If not, the timing of sex and final diapausing egg production of a species might not be optimized. For example, a low-density population of a rotifer species that still had growth potential could respond to the MIP produced by another co-occurring rotifer species. In this case, because investment in sex implies a decrease of the current growth rate, the population of a rare species could lose the opportunity to colonize the water column; on the other hand, males and

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sexual females of the rare species would be unlikely to encounter and mate. This hypothesis would imply differentiation in MIP among co-occurring congeneric species. However, besides this putative selection, other processes might oppose differentiation. First, within-species communication signals are expected to be under stabilizing selection because of positive frequency-dependent selection, in that a mutant having a different communication system would be selected against (Ryan and Wilczynski 1988; Brooks et al. 2005). Second, the premise of a low-density species with growth potential co-occurring with a high-density congeneric species might be rare. For instance, if the low-density species is an inferior competitor, it would not have growth potential.

Previous studies have provided mixed evidence with regard to the specificity of signals inducing sexual reproduction in monogonont rotifers of the genus *Brachionus*. On one hand, Gilbert (2003) found that an Australian strain of *Brachionus calyciflorus* was unable to induce sex in two American strains. The Australian strain is likely to be a sibling species of the American strains (Stelzer 2008), thus indicating species specificity of the inducing signal for sexual reproduction. On the other hand, Stelzer and Snell (2006) found that closely related, as well as more distant, species belonging to the *B. plicatilis* cryptic species complex could induce sex in each other. However, these authors studied long-term established laboratory populations that originally lived in geographical areas that are very distant from each other. These studies did not test crossed induction of sex between congeneric populations inhabiting the same region or pond. Yet this co-occurrence is a condition of the hypothesis of selection for differentiation in MIP. However, rotifer species are highly genetically structured, with important genetic differentiation among relatively close populations (Gómez et al. 2000, 2002). There are also signatures of local adaptation regarding sexual reproduction patterns (Campillo et al. unpubl.). As a result, local adaptation to co-occurrence with congeneric populations involving MIP differentiation cannot be ruled out a priori.

In the Iberian Peninsula, where the distribution and phylogeography of the *B. plicatilis* cryptic species complex has been well characterized (Gómez et al. 2002), at least four species are known to coexist from the Pleistocene to date, some of them occurring in sympatry (Gómez and Serra 1995; Gómez and Carvalho 2000; Ortells et al. 2000), along with extensive seasonal overlap (Carmona et al. 1995; Ortells et al. 2003). This offers an adequate scenario to test whether sex-inducing signals in the different species of the *B. plicatilis* species complex differentiate or not in sympatry. In terms of the cost of sexual reproduction, a higher differentiation in sexual reproduction–inducing signals leading to specificity might be expected to occur more among sympatric than allopatric populations. Moreover, specificity to the sexual reproduction–inducing signal could play a central role in maintaining reproductive barriers between closely related sympatric species.

In this study, the differentiation between rotifer congeneric species in their sexual reproduction induction signals was investigated by analyzing the capability of medium conditioned by a species population of the *B. plicatilis* species complex to induce sexual reproduction in a different species population also belonging to the complex. In case of complete differentiation between species, sexual reproduction levels in a species growing in a medium conditioned by a different species will not be significantly higher than in an unconditioned medium. If only partial differentiation has evolved, cross-induction will occur, but levels of sexual reproduction will be lower than in self-induction. To asses this topic, the study included both sympatric and allopatric populations.

Methods

Study sites, sampling, and clone foundation—We sampled the sediments of two ponds in eastern Spain—Torreblanca Sur (Cabanes-Torreblanca Marsh, Castellón; 40°9′N, 0°10′E) and Laguna de Salobrejo (Albacete; 38°55′N, 1°28′W; hereafter Salobrejo)—195 km distant, during May 2007 to collect diapausing eggs of local populations of different species belonging to the *B. plicatilis* species complex. Sediment handling and diapausing egg isolation protocol was as in García-Roger et al. (2006).

Fifteen clones each for the following populations were established: (1) *B. plicatilis* from Torreblanca Sur (hereafter *B.p.*TOS), (2) *Brachionus ibericus* from Torreblanca Sur (*B.i.*TOS), (3) *Brachionus rotundiformis* from Torreblanca Sur (*B.r.*TOS), (4) *B. plicatilis* from Salobrejo (*B.p.*SAL), and (5) *Brachionus manjavacas* from Salobrejo (*B.m.*SAL). All the clones were founded from diapausing eggs, except those of *B.i.*TOS. Because no individual of this population was found to hatch, we sampled the water column of this pond looking for 15 live animals. Taxonomic identification of species from Torreblanca Sur was based on morphology (Ciros-Pérez et al. 2001), whereas in the case of Salobrejo, the identification of *B. plicatilis* and *B. manjavacas* had to be based on the polymerase chain reaction–amplified cytochrome oxidase I (COI) mitochondrial gene and its analysis by restriction fragment length polymorphisms (RFLPs; Campillo et al. 2005). Despite the considerable past sampling efforts by the research group, no population of any other species belonging to the *B. plicatilis* species complex has ever been detected in the two sampled ponds. Stock cultures of the clones were kept in 20 mL of artificial seawater (Instant Ocean®, Aquarium Systems) at 12 g L^{-1} salinity, 18°C temperature, 35 μmol quanta m^{-2} s^{-1} constant light and were fed with an excess of the microalga *Tetraselmis suecica* (10^6 cells $mL^{-1} \approx 100$ mg C L^{-1}). The algae were cultured in f/2 enriched medium (Guillard and Ryther 1962) in the same conditions as the rotifers, except that the temperature was 25°C.

Experiment 1: Individual cultures—Crossed induction of sex was first studied with a two-way experimental design. This design yielded 25 combinations resulting from five conditioner populations and five test populations. The former were used to generate a medium conditioned by growing at high density, from which the latter were cultivated, and the level of sexual reproduction was measured.

For each combination, five randomly selected clones of each conditioning population were cultivated separately to avoid competition among them. Cultures were initiated by inoculating 100 asexual females of each clone into 2 liters of artificial seawater at what hereafter will be called standard conditions (12 g L^{-1} salinity, 25°C temperature, 35 μmol quanta m^{-2} s^{-1} constant light) with *T. suecica* at 10^6 cells mL^{-1} as food. Daily, a 5-mL sample from each of the five cultures was taken and fixed with Lugol (5%). Females in the fixed sample were counted and classified as sexual or asexual on the basis of the type of eggs carried, which allowed us to estimate both the population density and the mixis ratio (i.e., proportion of sexual females in the culture). The culture was allowed to grow until the seventh day, when sexual reproduction was known to be initiated, at which point we poured 400 mL of the culture of each conditioner clone for mixing into a 2-liter bottle. The 2-liter mixture of conditioned medium was centrifuged (12,000 × *g*, 20°C, 45 min) and microfiltered (successive filters of 30, 10, 1, 0.45, and 0.2 μm) to remove rotifers, algae, and detritus. In parallel, a *T. suecica* culture (*see* above for the culture conditions) was concentrated by centrifugation (1200 × *g*, 20°C, 5 min) to a low volume (<1 mL). This volume was added to the conditioned medium to reach a final food concentration of 10^5 cells mL^{-1}. A control medium was prepared by adding concentrated algae to artificial seawater at 12 g L^{-1}. This same procedure was repeated the eighth day of culture.

The effect of conditioned medium was tested in five clones for each test population, with two replicates and one control (growth in unconditioned medium) per clone by estimating the mixis ratio (i.e., proportion of sexual females) in the offspring. Before measuring the effect of the medium on the mixis ratio, special care was applied to control the pre-experimental conditions. If not, the response of the tested animals could be due to the environment of their mothers and grandmothers, rather than their own environment (i.e., the conditioned medium; Stelzer and Snell 2006). Hence, the test clones were grown (standard conditions; food, 10^5 cells mL^{-1} of *T. suecia*) for 3 d at densities less than 0.70 females mL^{-1} (i.e., the density threshold for sex induction in *B. plicatilis* according to Kubanek and Snell 2008). After these 3 d, pre-experimental cultures were established by picking up three asexual females that were allowed individually to grow in 50 mL of medium (standard conditions) and fed with *T. suecica* (10^5 cells mL^{-1}). Daily, their offspring were collected and cultured individually up to the third generation, from which the females to be used in the assays were finally separated. Three females per test clone were needed to initiate the experimental cultures: two for the two replicates plus one for the control. Whenever possible, the selection was made in a way that they did not share their mother, grandmother, or great-grandmother, although this criterion had to be relaxed because of some failures in reproduction (*see* Results).

Females to be tested were isolated in 25 mL of medium (conditioned medium or control) and kept in the medium for 2 d (medium renovated after 1 d). Their offspring was collected 24 and 48 h after the culture was started. Each descendent was isolated in a culture well (150 μL, standard conditions) and classified as asexual (i.e., if it produced a daughter) or sexual (i.e., if it produced a son or a diapausing egg), which allowed us to compute the mixis ratio. As a result of the experimental design, 375 assays were performed, resulting from 5 conditioner populations ×5 test populations × 5 test clones per population × 3 tested animals (i.e., two for conditioned medium + one for the control medium), and an average of 51.9 daughters per assay were classified as sexual or asexual (range, 8–91).

All statistics in this study were carried out with the use of R 2.6.1 statistical software (Ihaka and Gentleman 1996). Fisher's exact tests were used to compare the numbers of sexual to asexual daughters produced in conditioned and unconditioned media. Tests were one-tailed because the frequency of sexual daughters was expected to be lower in the controls than in the conditioned media (alternative hypothesis: true odds ratio < 1). Because multiple tests were performed, we used the Dunn–Šidák correction for statistical significance (Sokal and Rohlf 1995).

A generalized linear model (GLZ) on binary data of the counts of both types of offspring was used to study for significant effect of (1) the conditioner population (excluding controls because sexual reproduction was avoided there, *see* Results), (2) the test population, (3) the clone within test population, and (4) their interactions. We assumed a binomial distribution of data and logit as the link function. The goodness of fit of the model was assessed by the ratio between the deviance explained by the model with respect to the deviance of the null model (Nelder and Wedderburn 1972).

Experiment 2: Mass cultures—With the intention to increase the statistical power in our study, we performed a second experiment (Exp. 2) with mass cultures that was restricted to analyzing any effects suggested in the first experiment (Exp. 1). The effects of three populations (*B.p.*TOS, *B.p.*SAL, and *B.m.*SAL) were studied as conditioner and test populations in a two-way factorial design without replication, yielding nine experimental combinations. Replication was not used because of the intensive pre-experimental culturing required (*see* below). Because no sexual offspring were ever observed in the controls (unconditioned medium) in Exp. 1, we did not perform treatments without animals on the "conditioner" side. Only one clone was used per population, regardless of whether the population was a conditioner or a test population. The clones used were those that showed the highest mixis ratios in the stock cultures of each population tested.

For mass cultures, we used a 500-mL flask divided into two sides by a 30-μm Nytal mesh (Fig. 1). This mesh allows the flow of solutes and food but restricts the conditioner and test animals to their corresponding sides of the flask.

Before experimentation and similarly to Exp. 1, low-density and pre-experimental cultures (daily medium renewal for up to three generations) were established for both conditioner and test monoclonal populations to avoid early sex and to ensure that asexual females were used to start the experiment. Then, 10 asexual females from the

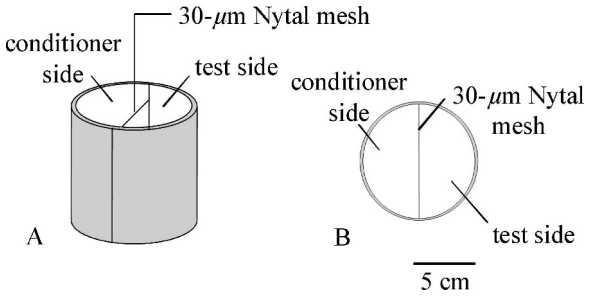

Fig. 1. Design of 500-mL PVC flask. The flask is divided into two equal compartments by a 30-μm Nytal mesh. (A) Overview. (B) Top view.

pre-experimental cultures of the conditioner population were allowed to grow for 9 d in the conditioner side of the 500-mL flasks at 25°C and 12 g L^{-1} salinity and were kept in darkness on an orbital shaker at low speed (40 revolutions per minute; Forma Scientific 416). Every day, rotifer density and mixis ratio were estimated by taking live samples of 10–50 mL from the conditioner side of the flask, which were returned to the culture after being counted. Algal density was also controlled daily and was estimated by measuring light extinction at 750 nm (Shimadzu uV-1603 spectrophotometer; Ciros-Pérez et al. 2004) and adjusted to $\geq$10 mg C L^{-1}. This ensured maximum growth rates in *B. plicatilis*. Food addition was carried out by adding a small volume of standard medium (<1 mL) with concentrated algae.

On the seventh day of culture, the conditioner population was expected to grow at high rates according to a previous study of these experimental clones (data not shown). On that day, 10 neonates belonging to the third generation of the pre-experimental culture of the test population were inoculated on the "test" sides of the experimental flasks. The offspring of these 10 females (75–95 females per experimental combination) were removed daily for 2 d, isolated individually in 150 μL of 12 g L^{-1} artificial seawater containing 10^5 cells mL^{-1} of *T. suecica*, and kept at 25°C and constant light (35 μmol quanta m^{-2} s^{-1}). After 2 d, their reproductive type was determined by the offspring produced (*see* Exp. 1), and the mixis ratio was computed.

Fisher's exact tests were used to test for differences in the sexual proportion of offspring of a test population when growing beside different conditioner populations. The alternative hypothesis was two-sided, and the Dunn–Šidák correction for multiple tests was implemented.

The counts of sexual to asexual females in the offspring were analyzed by GLZ assuming a binomial distribution of data and logit as the link function. This GLZ accounted for the effects of conditioner populations, test populations, and their interaction. Parameters in the underlying linear model were inspected to interpret the significant effects.

Results

Experiment 1: Individual cultures—Cultures of the conditioner populations varied in their average maximum density and mixis ratio (Fig. 2), with no significant correlation between mixis ratio and density over conditioner populations (Pearson's $r = -0.604$, $n = 5$, $p = 0.280$).

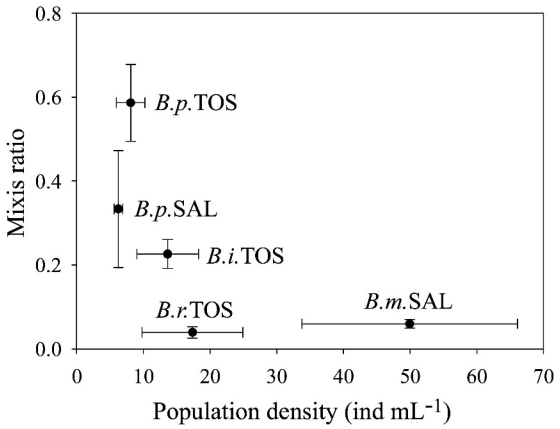

Fig. 2. Relationship between the mixis ratio and population density for the five conditioner populations studied. Data are averages ± SE.

During the three-generation pre-experimental culturing, some females were lost or removed because they were sexual (<10% of all data). These missing replicates always happened in the second generation. When available, they were substituted by the offspring from another replicate (i.e., a second-generation sister female). This could create dependence between replicates at the pre-experimental phase. However, the mixis ratios in the experimental cultures that came from pre-experimental cultures with three independent generations were not less similar than those coming from pre-experimental cultures with one independent generation. The respective variances were 0.029 and 0.088 ($F_{65,5} = 0.33$; $p > 0.75$).

Figure 3 shows the mixis ratio in the offspring of the five test populations after averaging the five clones per population, when assayed against the five conditioner populations. No sexual offspring were ever found in the controls. In contrast, the five populations tested responded by producing sexual offspring in the media conditioned by any population, with one exception (*B.i.*TOS in medium conditioned by *B.p.*TOS). Sex was observed in 24 of 25 combinations between conditioner and test populations, but after a one-tailed Fisher's exact test, significant differences with controls were found in only six clones (*see* Fig. 3). *B.m.*SAL only elicited a significant response on itself when compared with the control, which contrasts with the high density it reached in the culture to produce a conditioned medium (50 ± 16 individuals mL^{-1}). The response of *B.m.*SAL (as test population) to both *B.m.*SAL and *B.i.*TOS (as conditioners) exceeded the average mixis ratio observed in the *B.m.*SAL culture performed to prepare the conditioned medium. Interestingly, *B.p.*TOS responded significantly to the medium conditioned by either *B. plicatilis* population. Regardless of the statistical differences with controls, *B.p.*TOS showed rather similar mixis ratios when growing in media conditioned by any other population, whereas these mixis ratios were lower than the average mixis ratio observed in the *B.p.*TOS

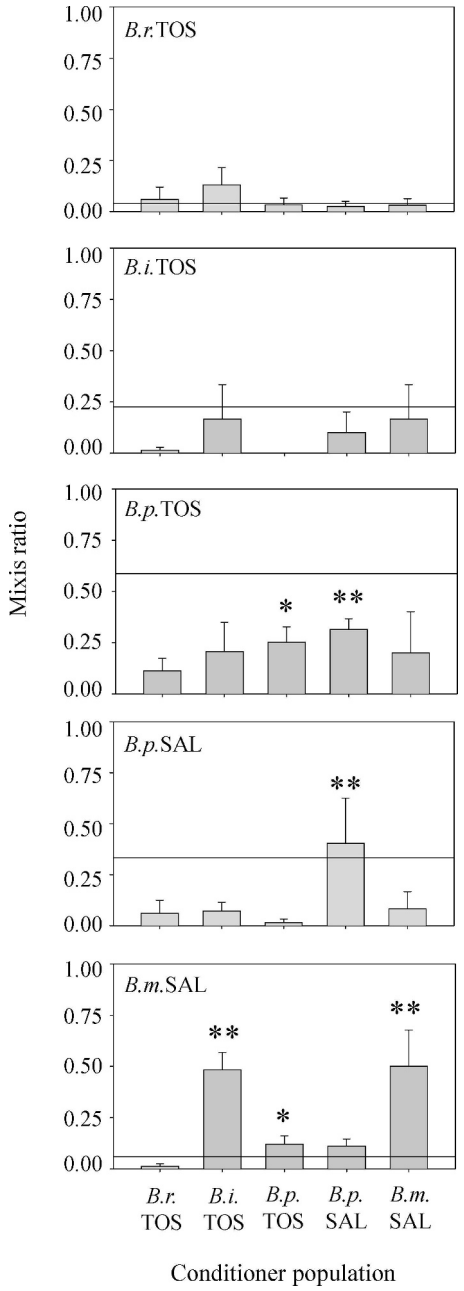

Fig. 3. Mixis ratio in the offspring of test populations when subjected to the media conditioned by five different populations. Data are averages ± SE. Horizontal lines are the average mixis ratio of five conditioner clones for each population. Asterisks indicate significant departure with respect to controls (Fisher's exact tests): * $0.05 > p > 0.002$ (lower limit is Dunn–Šidák's experimentwise error rate for 25 tests), ** $p < 0.002$.

culture used to prepare the conditioned medium. Contrasting with *B.p.*TOS, *B.p.*SAL responded significantly only to itself as conditioner.

Conditioned medium, if compared with control medium, did not have any effect on the fecundity of the females from which the mixis ratio was estimated (two-tailed *t*-test, $t = -0.235$, df = 297, $p = 0.814$). The average fecundity was 3.44 ± 0.02 neonates per female per 2 d. Moreover, mixis ratio and fecundity were not significantly correlated (Pearson's $r = -0.130$, $n = 299$, $p = 0.826$).

Table 1. Generalized linear model analysis on binary data of the counts of sexual and asexual offspring in Exp. 1. Model: binomial; link function: logit; % deviance explained: 76.1.

Effect	Deviance	df	*p*
Conditioner population (cond. pop.)	21.39	4	<0.001
Test population (test pop.)	32.41	4	<0.001
Cond. pop. × test pop.	55.27	16	<0.001
Clone (test pop.)	60.31	24	<0.001
Cond. pop. × clone (test pop.)	52.49	59	0.713

A GLZ on the mixis ratios measured in conditioned cultures (Table 1) showed the existence of significant effects from the conditioner population and the test population, as well as an effect from the interaction between test and conditioner populations. There were also differences among clones within a population. Some of these clones responded to the conditioned media and produced sexual offspring, whereas others did not. Notwithstanding, a significant interaction between clones within one population and the conditioner population was not detected.

Experiment 2: Mass cultures—The dynamics of the population density and mixis ratio of the monoclonal conditioner cultures of *B.p.*TOS, *B.p.*SAL, and *B.m.*SAL are shown in Fig. 4. *B.p.*TOS during the period the test population was growing in the other side of the flask (i.e., the last 2 d of culture) showed an average density of 10.4 ± 0.9 individuals mL^{-1} and an average mixis ratio of 0.83 ± 0.08. These parameters were 20.7 ± 1.9 individuals mL^{-1} and 0.35 ± 0.06 for *B.p.*SAL, and 23.4 ± 1.7 individuals mL^{-1} and 0.34 ± 0.05 for *B.m.*SAL; thus, the latter showed the highest density and the lowest mixis ratio.

The mixis ratio in the test populations, determined as the proportion of sexual offspring from individually isolated females, is shown in Fig. 5. *B.p.*TOS responded to itself and *B.p.*SAL as conditioners, so in these test cultures, *B.p.*TOS reached similar mixis ratios to those found in the conditioner culture of *B.p.*TOS. In contrast, the mixis ratio of *B.p.*TOS when growing with a rather dense culture of *B.m.*SAL was lower than those referred to above. Accordingly, significant differences were found in the response of *B.p.*TOS to the three conditioner populations (two-tailed Fisher's exact test, $p < 0.001$). *B.p.*SAL responded similarly to itself and to *B.p.*TOS as conditioners, also reaching in these test cultures the same mixis ratio as when acting as conditioner. Conversely, no sexual offspring were observed in *B.p.*SAL when cultured with its sympatric *B.m.*SAL. A two-tailed Fisher's exact test found significant differences in the response of *B.p.*SAL to the different conditioner populations ($p < 0.001$). The differences among the mixis ratio of *B.m.*SAL when growing with the three different conditioner populations were not significant (two-tailed Fisher's exact test, $p > 0.5$), and the observed mixis ratios were always less than, but close to, the mixis ratio observed in *B.m.*SAL when grown as a conditioner.

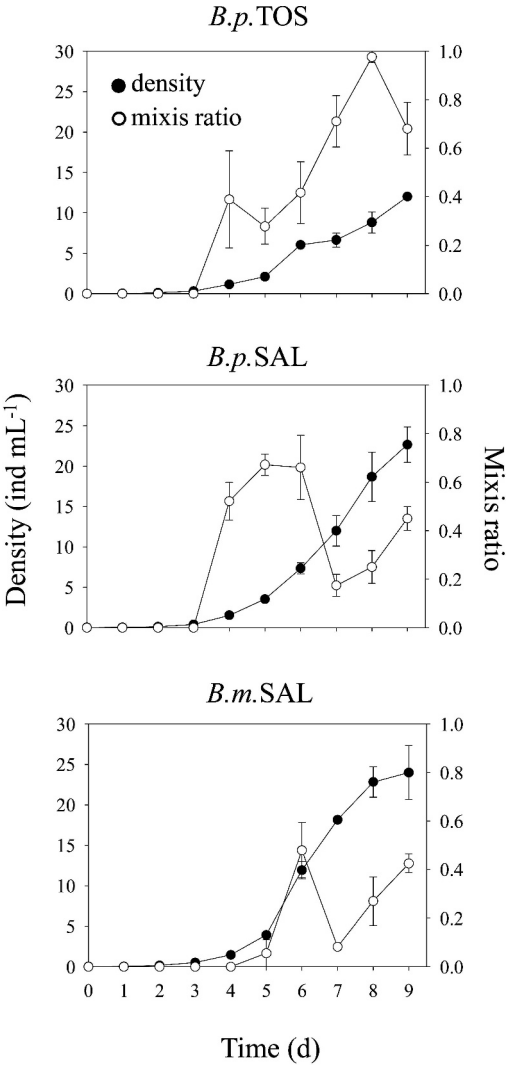

Fig. 4. Density and mixis ratio dynamics of conditioner population cultures.

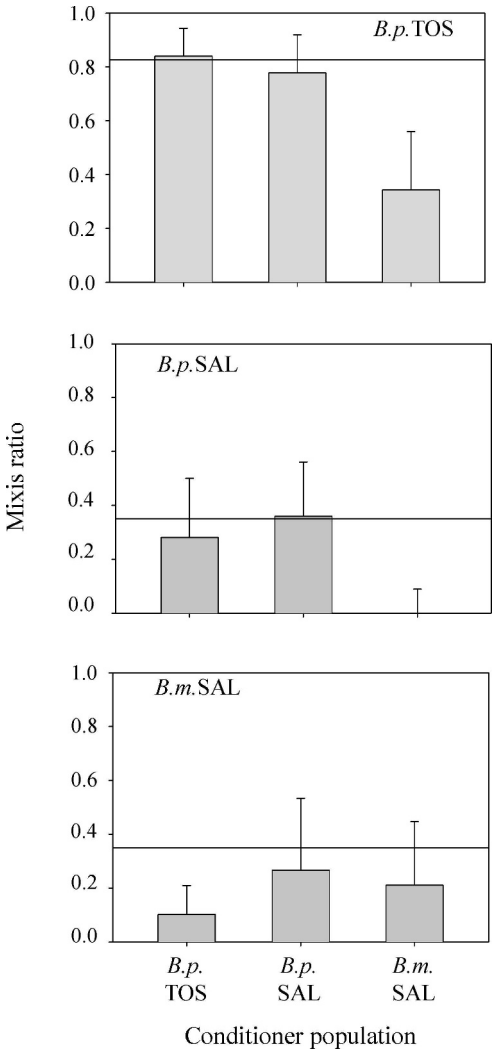

Fig. 5. Mixis ratio in the offspring of test populations when cultured with the same three populations used as conditioners. Bars indicate 95% confidence interval for proportions (Rohlf and Sokal 1995). Horizontal lines indicate the mixis ratio of each conditioner population.

A GLZ on the mixis ratio revealed the existence of significant effects of (1) the test population, (2) the conditioner population, and (3) the interaction between the test and conditioner populations (*see* Table 2).

These three effects were investigated by computing coefficients in a linear model, so that the contribution of the levels of each factor (test population, conditioner population, and their combinations) could be estimated. *B.m.*SAL had a low effect as conditioner (''conditioner population'' effect; Fig. 6) and *B.p.*TOS had in general a high response (''test population'' effect; Fig. 6). The contributions resulting from interactions, estimated as deviations from a linear model including only the main effects, are shown in Table 3. They reveal a preference for self-induction (i.e., positive interaction when the same population is assayed as conditioner and test). Moreover, both populations of *B. plicatilis*—wherever their origin—had negative interaction coefficients when *B. manjavacas* was the conditioner, whereas *B. manjavacas* had negative interaction coefficients when any *B. plicatilis* population was the conditioner. Also noticeable was that the interaction coefficients were lower between allopatric than between sympatric populations.

Discussion

In this study, the process of crossed induction of sex in monogonont rotifers was investigated for the first time with

Table 2. Generalized linear model analysis on binary data of the counts of sexual to asexual offspring in Exp. 2. Model: binomial; link function: logit; % deviance explained: 99.9.

Effect	Deviance	df	*p*
Conditioner population (cond. pop.)	15.60	2	< 0.001
Test population (test pop.)	57.18	2	< 0.001
Cond. pop.. × test pop.	15.98	4	0.003

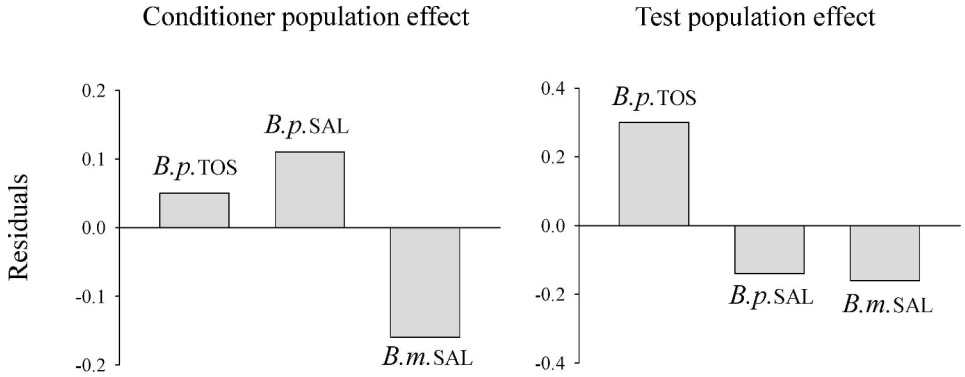

Fig. 6. Deviation residuals on the ratio of sexual to asexual offspring associated with main effects from the linear model implemented in Exp. 2.

the use of sympatric and allopatric congeneric populations. Previous studies were based on allopatric strains (Gilbert 2003; Stelzer and Snell 2006; Stelzer 2008), so they did not test whether local adaptation to co-occurrence with congeneric populations resulted in between-species differentiation of the signals for sex induction. Our results suggest that the populations in the studied species complex responded to the signals produced by other species in the complex. Despite only 6 of 25 cross-induction tests revealing mixis ratios significantly greater than in the controls, the total absence of sex in the controls contrasts strikingly with the observation of sex in all but one culture conditioned by the previous growth of any other species. This result indicates that, at least for these species, there is little differentiation in sex induction signal (i.e., MIP). Previous studies have provided mixed evidence with respect to the specificity of response to sex-inducing signals in monogonont rotifers of the genus *Brachionus* (Gilbert 1963, 2003; Stelzer and Snell 2006; Stelzer 2008). Whereas in the *B. plicatilis* species complex, no specificity was found among allopatric populations of different species of the complex (Stelzer and Snell 2006) and other taxonomic groups (e.g., *Artemia salina*; Carmona et al. 1993), studies in *Brachionus calyciflorus* are more controversial (Gilbert 2003; Stelzer 2008). On one hand, Gilbert (2003) found specificity in the response of two American strains to one Australian strain of *B. calyciflorus*. However, the taxonomic status of these strains is unclear because they are morphologically different and behave as distinct species (e.g., they show mating isolation). More recently, Stelzer (2008) has reported that a European strain and an American strain of this species are effectively able to induce sex in each other, but to date, no study has included sympatric populations, which is relevant to test for the specificity hypothesis

Table 3. Deviation residuals of the ratio of sexual to asexual offspring because of the conditioner population × test population interaction effect.

	Conditioner population		
Test population	*B.p.*TOS	*B.p.*SAL	*B.m.*SAL
*B.p.*TOS	0.13	0.01	−0.14
*B.p.*SAL	0.02	0.03	−0.05
*B.m.*SAL	−0.15	−0.04	0.19

We encountered some difficulties in this study. First, although our design in Exp. 1 was useful to test for an effect of conditioned media on sexual reproduction of test populations, it did not allow us to study more precisely whether the response of a test population varied depending on the conditioner population. A direct analysis of such an interaction would need an ad hoc test with low statistical power because of the loss of degrees of freedom. Note that a great effort was made during the pre-experimental phase to avoid early sex induction by density and to control for environmental maternal effects. These measures made it impossible to increase the number of experimental replicates and the degrees of freedom needed for further analyses. Notwithstanding, we solved this problem by means of the mass culture approach performed in Exp. 2, which was specifically designed to explore the above-mentioned interactions. An additional difficulty in Exp. 1 was the management of sister female replicates from the pre-experimental phase (<10% of all data; *see* Results). Luckily, their variance was greater than that of three independent-generation females, so they were included in our analyses without a decrease in sample size. Finally, we observed that some test clones exhibited a higher response than the average mixis ratio of conditioner clones of the same population. We believe that this finding is explained by our experimental design, which included different clones used both as test and conditioner animals in each population to assess for within-population variation in the response to the different conditioned media.

Our two experiments showed variation among populations in their average mixis ratios. The GLZs of both experiments detected a significant effect for the test population. For instance in Exp. 1, *B. rotundiformis* responded very weakly to the conditioned media. This species also had a low mixis ratio when cultured to produce the conditioned medium, although this could be due to a low conditioning capability (i.e., low production of MIP). Previous studies suggest that *B. rotundiformis* has in general lower mixis ratios and needs higher population densities to induce sexual reproduction than *B. plicatilis* (Carmona et al. 1995). Moreover, the significant effect of the test population found in Exp. 2 is associated with the response

of *B.p.*TOS, with a higher coefficient than that of the other populations (Fig. 6). These results suggest that different populations of the *B. plicatilis* species complex have different thresholds for the concentration of MIP needed to induce sex (Snell and Boyer 1988; Carmona et al. 1995; Snell et al. 2006) or have different mixis ratios when sex is induced. We return to this issue later.

A part of the variation in the response to the conditioned media can be assigned to differences in the capability of the populations to condition the medium, that is, to produce MIP. In Exp. 2, the significant effect of the conditioner population is associated with differences between *B.m.*SAL, with relatively low conditioning capability, and the other populations. Statistical analysis of the results of Exp. 1 also detected a significant effect of the conditioner population. Associated with this result is the lack of any significant effect produced by the medium conditioned by the *B.r.*TOS population. This could be because of the biomass in the conditioning culture. *B. rotundiformis* belongs to the smallest morphotype described in the *B. plicatilis* species complex (Ciros-Pérez et al. 2001). It is ~50% the volume of *B. plicatilis* (Ciros-Pérez et al. 2001). Hence the per capita production rate of MIP might be low enough not to induce sex in other populations despite a density that was higher than in many of the other populations studied (Fig. 2).

Differences in MIP production and responsiveness to MIP (i.e., MIP threshold for sex induction and the mixis ratio after induction) seem to explain a significant part of our results. It has been proposed that the optimal pattern of sexual reproduction in monogonont rotifers depends on environmental uncertainty (Serra and King 1999). Sexual reproduction in populations inhabiting high-uncertainty environments should be expected to be induced early—by either high MIP production or high responsiveness to MIP—to protect against unpredictable years with short planktonic growth periods. According to theoretical expectations, Carmona et al. (1995) found that sympatric populations of congeneric *B. plicatilis* and *B. rotundiformis* showed different patterns of sexual reproduction related to habitat uncertainty. *B. plicatilis* showed a continuous sexual reproductive pattern and a lower MIP threshold for sex induction. This is considered to be optimal if habitat uncertainty is high, in that diapausing egg production is guaranteed by starting sex as soon as possible (i.e., a bet-hedging strategy). On the other hand, *B. rotundiformis* had a punctuated pattern of sex and a higher MIP threshold that would be optimal in predictable environments, with sex taking place at the end of the suitable period. This finding fits well with our results. Moreover, a recent study by Campillo et al. (unpubl.) has shown that life history traits related to sexual reproduction (e.g., production of male eggs, male production and diapausing egg production) differ among *B. plicatilis* populations from different habitats. Interestingly, populations with high values in sex-related life history traits occupied the more uncertain habitats. Indeed, the finding of variation in the within-population level suggests the existence of heritability for the evolution of sexual reproduction patterns.

However, we also found interactive effects, so some responses cannot be explained entirely as the result of differences in average responsiveness to MIP, in MIP production, or in both. Interestingly in Exp. 1, four of six significant responses occurred when the medium was conditioned by the test population (i.e., self-induction), whereas *B. manjavacas* responded significantly to one *B. plicatilis* population, these two species being phylogenetically the most closely related populations included in our study (Gómez et al. 2002). This pattern suggests a relationship between the level of differentiation in the signal that induces sex and phylogenetic distance. Moreover, the interaction coefficients found after applying a linear model to the results of Exp. 2 (Table 3) are positive when the conditioner and test populations are the same and negative otherwise. This suggests some degree of differentiation in MIP between *B. manjavacas* and *B. plicatilis*. The absolute values of these coefficients are higher for allopatric populations, so that local adaptation involving increased differentiation in MIP seems not to have happened.

Globally, besides differences in the response and production in MIP, our results are consistent with a slight differentiation in MIP. A slight differentiation implies that, when two congeneric species co-occur in the water column, a species, even at low density, would respond to the density of the other species producing sexual females. This has three important potential implications. First, species boundaries are unlikely based on temporal segregation of sexual periods and should be maintained mainly by other mechanisms, such as the proposed sex–mate pheromone recognition system (Snell et al. 1995; Snell and Stelzer 2005; Kubanek and Snell 2008). Second, because sex depletes growth rate (Maynard Smith 1978), it would be difficult for a population with low density, but responding to the MIP of another congeneric population, to increase. Third, such a low-density population could fail to produce diapausing eggs because of low male–female encounter probability (Snell and Garman 1986). These two latter points prompt the question of why a deeper MIP differentiation has not evolved in these rotifer species.

Differentiation of the signals for sex induction could be a slow process, even between sympatric congeneric populations. On one hand, traits involved in individual communication could be under strong stabilizing selection (Ryan and Wilczynski 1988). On the other hand, in some rotifer species of the genus *Brachionus*, the timing of sexual reproduction is partially controlled by an endogenous, transgenerational factor (Gilbert 2002; Schröder and Gilbert 2004). Stem females hatching from diapausing eggs and females produced in the next several parthenogenetic generations have a significantly lower propensity to respond to crowding, and so to MIP concentration, than females from later parthenogenetic generations, which is a phenomenon that guarantees clonal growth for late-hatching clones (Serra et al. 2005). Stelzer and Snell (2006) have proposed that delayed sex by means of this transgenerational effect could be a mechanism that reduces selection pressure for adaptive differentiation of the sex-inducing signal.

Nevertheless, the absence of any strong specificity in sex induction in sympatric populations, as shown by our

results, could imply an adaptive advantage if the concentration of MIP produced by an abundant population of a species would be a cue for the deterioration of the environment of a rare congeneric population of a species. Interestingly, Ciros-Pérez et al. (2002), working with three species of the *B. plicatilis* complex, found a negative association between competitive ability and sex investment and suggested that sex, needed to produce diapausing eggs, was a way to escape competition. For a rotifer population going into extinction in the water column, sex, even having a low probability of being successful, should be better than maintaining parthenogenetic reproduction. In this interpretation, the hypothesis that the production of MIP as an anticipated signal of habitat deterioration caused by resource overexploitation by either the density levels of a population or its competitors, gains strength to drive the evolution of the timing of sex (Carmona et al. 1993; Serra and Carmona 1993; Serra et al. 2004).

The unspecific effect of MIP among sympatric and allopatric congeneric populations of the *B. plicatilis* species complex shown by our results suggests that (1) differentiation of sex-inducing signals is a slow process, likely constrained by both environmental and endogenous factors; (2) the responsiveness to the environmental MIP—whatever species it comes from—is related to a strategy to escape an adverse competitive habitat; and (3) rotifer species vary in their responsiveness and production of MIP, which could be related to differences in their optimal patterns of sexual reproduction. A deeper understanding of the phenomenon could be obtained by studying the interaction between sexual reproduction and interspecific competition, particularly by focusing on the interaction between *B. plicatilis* and *B. manjavacas*.

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