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Induction of sexual reproduction in *Brachionus plicatilis* (Monogononta, Rotifera) by a density-dependent chemical cue

Abstract—Induction of mixis (sexual reproduction) in rotifers of the genus *Brachionus* is believed to be triggered by a chemical that is released into the water and accumulates at high population densities. However, direct and conclusive evidence for this hypothesis is thus far lacking. In this study, two mass cultures of the rotifer *Brachionus plicatilis* were monitored as they grew from low to high population densities. Conditioned water was prepared daily from these cultures, and females were exposed in a bioassay, which consisted of juvenile *Brachionus,* cultured individually in large volumes that would normally suppress mixis. Conditioned water induced mixis in the bioassay at rates comparable to those found in the mass cultures. Both in bioassay and mass cultures, mixis was essentially absent in the beginning of the experiment, when population densities were very low. The first mictic females appeared at densities of 0.1 females ml^{-1}, and their proportion increased rapidly as the populations grew to >1 female ml^{-1}. The maximum rates of mixis in the bioassay were highly significant and reached 51% of those observed in the mass cultures. These results strongly support the hypothesis that mixis in *Brachionus plicatilis* is induced by a density-dependent chemical cue.

Chemical signals, exchanged on the intraspecific level are an important means of communication in many aquatic invertebrates (Larsson and Dodson 1993; Snell 1998). For example, sex pheromones permit individuals to locate or recognize partners of the opposite sex (Miyake and Beyer 1974; Stanhope et al. 1992; Snell et al. 1993). Chemicals that accumulate with increasing population density (crowding chemicals) provide information about the likelihood of male–female encounters. Such information is particularly important for species with small perceptive volumes, such as many invertebrates, which depend on chance encounters for mate location. The ability to sense conspecific crowding chemicals has been reported for many zooplankton species (Kleiven et al. 1992; Carmona et al. 1993; Burns 1995; Yoshinaga et al. 1999).

In monogonont rotifers of the genus *Brachionus*, a chemical cue that induces the transition from asexual to sexual reproduction has been hypothesized. The adaptive significance of both parts in the life cycle of these cyclical parthenogens is well established (Serra and King 1999; Serra et al. in press). Asexual reproduction allows rotifers to build up large populations rapidly, whereas sexual reproduction is, for most monogononts, the only way to produce a dormant stage that can survive adverse environmental conditions. Other more general advantages of sexual reproduction might apply for monogonont rotifers as well (West et al. 1999).

The sexual part of the rotifer life cycle is initiated by the production of mictic females (Wallace and Snell 2001). If mictic females are not fertilized, they produce haploid males. However, if they are fertilized, they produce female offspring that are developmentally arrested in an early embryonic stage (resting egg) and can remain dormant for up to several years. The biotic and abiotic factors that can cause amictic (asexual) females to produce mictic daughters have been studied since the early days of rotifer biology. So far, the only chemical identified that triggers mixis is dietary alpha-tocopherol in the genus *Asplanchna* (Gilbert 1980).

Mixis induction in the genus *Brachionus* is density dependent (Gilbert 1977). At low population densities, females reproduce asexually. As the population grows above a critical density threshold, mictic females appear and sexual reproduction is initiated. Gilbert (1963) was the first to propose the hypothesis of chemical induction for *Brachionus* where mixis is induced by chemicals released into the water. Gilbert's conjecture was based on autoconditioning experiments with the freshwater rotifer *Brachionus calyciflorus.* He showed that individual females cultured in small volumes self-induce mictic offspring at rates comparable to females cultured together. Self-induction has been demonstrated in other *Brachionus* species as well (Hino and Hirano 1976; Carmona et al. 1993). An additional experiment of Hino and Hirano (1976) demonstrated that mixis in a high-density culture of *Brachionus plicatilis* can be suppressed by frequent replacement of the culture medium.

All the above studies provided strong, yet indirect evidence for chemical induction of mixis. However, the ultimate proof for chemical induction is the demonstration that conditioned water from a high-density culture can by itself induce the production of mictic daughters. So far, this has not been convincingly demonstrated in rotifers. In this study, we followed two mass cultures of *Brachionus plicatilis* as they grew from low to high population densities. We applied conditioned water taken daily from these cultures to a bioassay of individually cultured females and tested its ability to induce production of mictic daughters. We demonstrate unequivocally that a density-dependent chemical factor induces mixis in *Brachionus plicatilis.*

Materials and methods—Two clones of *Brachionus plicatilis* (designated IR1 and IR2) were hatched from resting eggs collected in June 2001 from a small lagoon at Indian Rocks Beach, Florida, on the western side of Tampa Bay. Rotifers were cultured in 18‰ artificial seawater (Instant Ocean®) enriched with F nutrients (Guillard and Ryther 1962) and fed the green alga *Tetraselmis suecica.* Stock cultures of IR1 and IR2 were maintained in 20-ml test tubes at 18°C, and new cultures were inoculated every 7–10 d with three amictic females. This combination of temperature and transfer schedule kept mixis rates low and effectively prevented sexual reproduction. Two weeks before the experiments, populations were acclimated to the experimental temperature of 25°C.

Mass cultures of IR1 and IR2 were initiated by inoculating two 120-liter tanks, each with 1,000 individuals of one clone. At the start of the experiment, algal concentrations were adjusted to 220 cells μl^{-1} by adding *Tetraselmis* from a high-density culture. Each tank was continuously illuminated by two 20W fluorescent bulbs. During the first days, while rotifer densities were low, algae were growing at a rate of $\sim 0.25\ d^{-1}$. Each tank was aerated through two glass tubes reaching to the bottom of the tank.

Rotifers were sampled from the surface after the tank had been stirred thoroughly with a paddle. In the beginning of the experiment, when rotifer densities were low, 50-μm mesh plankton sieves were used to concentrate the samples. Sampling volumes varied from 5 ml to 6 liters depending on the rotifer density of the cultures. On average, 150–500 animals were sampled per tank.

Mixis rates in the mass cultures were estimated from a sample of 96 young, non–egg-bearing females that were isolated individually in 48-well polystyrene plates, each well filled with 500 μl of seawater (salinity = 18) containing *Tetraselmis* at a density of about 500 cells μl^{-1}. Two days later, their reproductive type was determined by the offspring that they produced. Females were classified as mictic if they produced males or resting eggs or amictic if they produced females. The remaining animals were fixed in Lugol's solution, and densities were estimated from counts using a Sedgewick–Rafter chamber (Wildlife Supply Company). Algal concentrations in the mass cultures were estimated from photometric measurements by a previously established regression of *Tetraselmis* concentration versus photometric extinction (at 800 nm wavelength).

Conditioned water was prepared daily from culture water of both tanks. About 600 ml of water was taken from each culture, and rotifers were removed with a 50-μm sieve. After 45 min of centrifugation at 12,000 $\times$ g, the supernatant was carefully decanted and filtered through a 0.2-μm Nylon membrane. About 250 ml of the conditioned water was stored at 4°C for incubations on the second day (*see Experimental exposures*).

Low-density precultures: To provide amictic juveniles for the bioassay, females of the IR1 clone were cultured individually in 25-ml polystyrene petri dishes at food concentrations of 500–1,000 cells μl^{-1} *Tetraselmis* with daily transfers into fresh medium. The precultures consisted of 15 adult animals, 1–2 d old, which produced juveniles for the experimental exposures, and 15 juvenile animals, 0–1 d old, which served as ''adults'' on the following day. Under these conditions, the IR1 clone reproduced exclusively asexually. No mictic females were observed during the entire 10 d of the preculture.

Experimental exposures: Each day, 8–10 juveniles were exposed individually to one of the following experimental treatments: water from tank 1 (conditioned water by clone IR1), water from tank 2 (conditioned water by clone IR2), or fresh F2 medium (negative control). Positive controls were obtained by placing juveniles individually into 1-ml F medium in 24-well plates without exchange on the second day. Thus, these rotifers were exposed to autoconditioned medium. In all experimental exposures, algal concentrations were adjusted to 500–1,000 cells μl^{-1} by adding *Tetraselmis* from a concentrated culture.

Exposures to conditioned medium were similar to the precultures. Juveniles were individually exposed to conditioned medium in 25-ml petri dishes for 2 d. On the second day, they were transferred into conditioned medium that had been stored at 4°C from the previous day. During this 2-d period, females usually produced at least three or four daughters. Those were removed daily, and their reproductive type was determined in the same way as described for the mass cultures.

Mixis rates were calculated as the percentage of mictic females out of the total number of females examined. Statistical analysis was performed on absolute frequencies. Because of the small sample sizes and often sparsely populated cells in the frequency tables, Fisher's exact test (two-tailed) was used. The appropriate use of this test under conditions where only one set of the marginal totals is fixed (as in our case) has been demonstrated by Tocher (1950).

Results—Mass cultures: The rotifer populations in the mass cultures grew exponentially during the first half of the experiment and thereafter gradually approached maximum densities of 24 individuals ml^{-1} in clone IR1 and 52 individuals ml^{-1} in clone IR2 (*see Fig. 1, top panels*). During the first 4 d, when reproduction was asexual, population growth rates of both clones were very similar: $r = 1.25\ d^{-1}$ in clone IR1 and $r = 1.33\ d^{-1}$ in clone IR2. The first mictic females were found in juveniles sampled from the clones on day 3 and day 4, respectively. The actual time of mixis induction, however, must have happened about 1 d earlier when these juveniles were in an early embryonic stage. Thus, the first induction of mixis corresponds to population densities of about 0.1 rotifers ml^{-1} (*see Fig. 1, top panels*). The highest levels of mixis were 98% in clone IR1 and 62% in clone IR2 (*see Fig. 1, middle panels*). In both clones, mixis levels declined toward the end of the experiment as populations entered a stationary phase of population growth.

Bioassay: From days 2–9 of the experiment, conditioned water was prepared from both mass cultures and tested for the ability to induce mixis in individually cultured females. No mictic females were observed in the negative controls, which were females cultured individually in 25 ml of F medium. The first mictic females in the bioassay were observed on day 3 in animals treated with water from tank 1 and on day 2 in animals treated with water from tank 2 (*see Fig. 1, lower panels*). However, throughout days 2–5, mixis rates were too low to be statistically different from the controls ($p > 0.05$, Fisher's exact test). From days 6–9, conditioned water from both tanks significantly induced mixis in bioassay test animals ($p < 0.05$ and $p < 0.001$, Fisher's exact test, *see* Fig. 1, lower panels). Mixis rates were determined on days 2–8 in the positive controls, which were individual females cultured in 1 ml F medium. Self-induction of mixis fluctuated around a mean of 36% and was always significantly higher than the negative control ($p < 0.05$ and $p < 0.001$, respectively, Fisher's exact test).

Individual females treated with conditioned water pro-

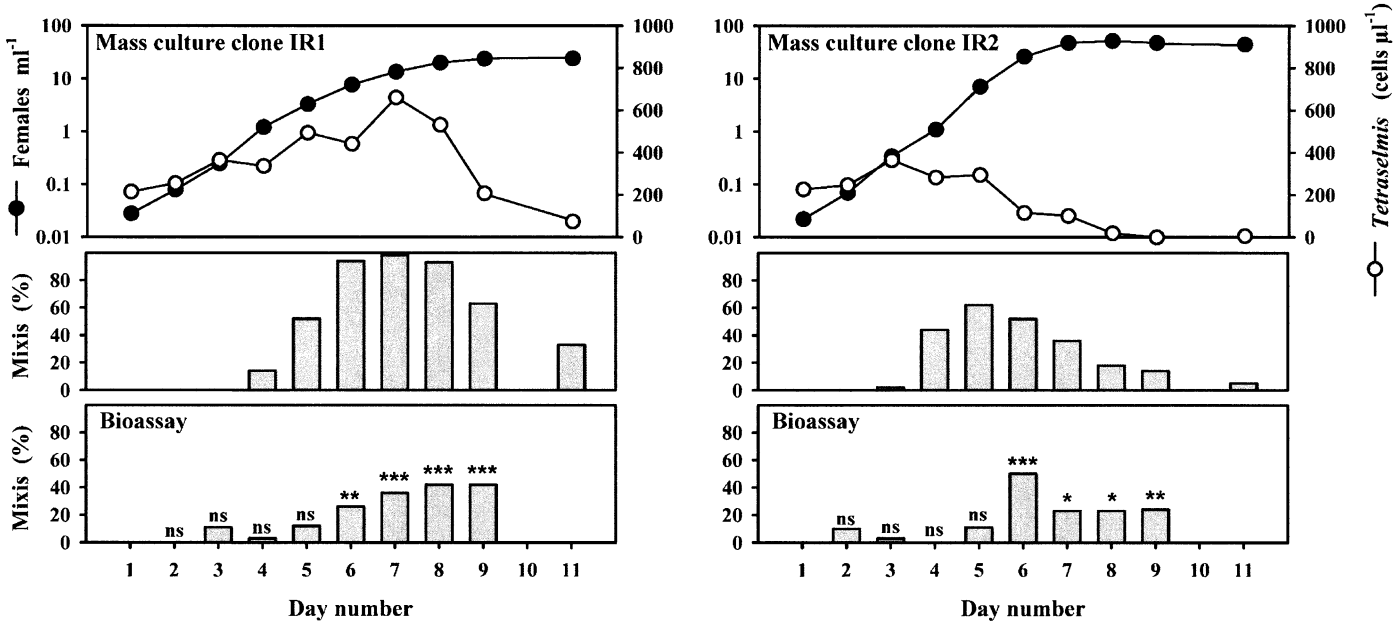

Fig. 1. Mixis in mass cultures with clones IR1 and IR2 and bioassays with clone IR1. (Top) Rotifer population density and algal concentrations in the mass cultures. (Middle) Mixis in the mass cultures. (Bottom) Mixis in the bioassays, which used conditioned water taken from the mass culture on days 2–9. Statistical significance of mixis in the bioassay was tested using Fisher's exact test: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; ns, not significant ($p > 0.05$).

duced both amictic and mictic daughters. Hence, the observed mixis rates did not occur because some females produced only amictic offspring whereas others produced only mictic offspring. Females treated with conditioned water also did not show any age-related patterns in the production of mictic daughters. The proportions of mictic offspring harvested after the first versus the second day of the bioassay were not significantly different ($p = 0.831$, $\chi^2_1 = 0.05$, $n = 623$).

Discussion—Only one report has been published concerning the induction of mixis in individually cultured females with conditioned water (Carmona et al. 1993). This publication is often cited as evidence for chemical induction in *Brachionus* (e.g., Snell 1998); however, less than 1% of the offspring of mothers treated with conditioned water became mictic, despite up to 70% mixis in this clone in mass cultures. Carmona et al. (1993) compared mixis rates in offspring of treated versus control females using a *t*-test and found their results to be significant. Given their categorical data and large sample size, a chi-square test with Yates correction probably would have been more appropriate, but when we applied this test to their data (*see* p. 150, table 2 in Carmona et al. 1993), it yielded a nonsignificant result ($p = 0.0784$, $\chi^2_1 = 3.1$, $n = 1{,}079$).

The results of our study provide convincing data for the model of chemical induction of mixis in *Brachionus.* Both the experimental animals in the bioassay and those of the mass cultures showed similar patterns of mixis throughout the course of our experiment. In the beginning of the experiment, sexual reproduction was essentially absent from mass cultures, but increased strongly with population density. The maximum rates of mixis in the bioassay were highly significant and reached 51% of those in the mass cultures. Because no mictic females were ever found in the negative controls, these results demonstrate that mixis was induced exclusively by a chemical factor. Physical contacts among females are not needed to induce mixis because the chemical stimulus in conditioned medium was sufficient.

Theoretically, one should expect mixis rates in the bioassay to be exactly the same as those in the mass cultures. Two reasons might explain why the bioassay usually yielded lower values. First, because the bioassay captured just a part of the individual's life history (2 d instead of the average 8–10 d total lifetime of a *Brachionus* female at 25°C), it might underestimate the mixis rate. It is possible that prolonged exposure to the mixis factor, even if at very low concentrations, enhances the physiological response of individuals. In addition, transgenerational effects, such as daughters responding more strongly to the mictic inducing factor if their mothers had already been exposed, are conceivable. Transgenerational effects with regard to the production of resting eggs have been demonstrated for *Daphnia* (Alekseev and Lampert 2001; LaMontagne and McCauley 2001). Second, the mixis inducing factor could be chemically unstable or volatile. Its activity could have been diminished during the preparation of the conditioned water, perhaps because of adsorption onto the membrane filter or the walls of the petri dishes.

The factors that induce sexual reproduction and the formation of resting stages have been studied in detail in another cyclical parthenogen. In *Daphnia,* the production of males can be induced by either low food, photoperiodic cues, or crowding chemicals (Stross and Hill 1965; Kleiven et al. 1992). However, the simultaneous action of these three stimuli is usually required for successful resting egg formation

(Kleiven et al. 1992). Photoperiodic cues appear to be more important in *Daphnia* clones that originate from higher latitudes, where seasonal changes are more predictable (Stross 1969). The only rotifer in which photoperiodic cues have been demonstrated to induce mixis is *Notommata* (Pourriot and Clement 1981). It is conceivable that photoperiod could also have an effect in *Brachionus plicatilis* clones that originate from temperate regions. However, in our clones from Florida, this stimulus was obviously not required.

In our mass cultures, algal concentrations changed from high to low during the experiment because of grazing by rotifers. It could be argued that this introduces a confounding variable (algae concentration) in addition to the change in rotifer density. The only way in which such changes could have confounded our results would be by a chemical factor from the algae that suppresses mixis. This factor would be abundant at high algal concentrations (in the beginning of the experiment) and scarce at low algal concentrations (at the end of the experiment). However, this hypothesis can be excluded on several grounds. First, test animals were exposed to the conditioned water were cultured at five times higher algal concentrations than those in the early mass cultures. If the above-mentioned algal factor were real, it should have prevented mixis in our bioassay. Second, animals in the positive controls (self-induction treatment) were exposed to similarly high food concentrations, yet mixis was induced throughout the whole experiment. In fact, published results suggest that low algal concentrations actually suppress rather than enhance the mictic response of rotifers, probably because of a nutritional effect (Snell 1986; Snell and Boyer 1988). Finally, in both mass cultures, periods of maximal mixis induction broadly overlapped with periods when the algae concentrations were still high (*see Fig. 1, top panels*); therefore, it seems obvious that a rotifer chemical, rather than an algal chemical, is responsible for the observed pattern of mixis.

The exact timing of mixis induction during ontogenesis is unclear, but it seems to happen very early in the development of individuals that eventually become mictic (*see* references in Gilbert 1963). The current model is that the mixis chemical is sensed by the amictic mother, stimulating her to produce mictic offspring (Gilbert 1963). This process seems to be reversible because Hino and Hirano (1976) demonstrated that individual mothers can switch back to producing amictic offspring as soon as the renewal rate of the medium is increased (i.e., the concentration of the mixis chemical is decreased). This suggests that the constant production of mictic offspring requires a constant presence of the mixis chemical. One possibility of how the mother releases the developmental cascade that results in mictic offspring would involve cytoplasmatic factors in the vitellarium. Such factors might be produced by the mother upon receiving a stimulus through the mixis chemical and would be passed to the developing egg along with the cytoplasm during oogenesis (Bentfeld 1971; Stelzer 2001). Working with conditioned medium for controlled induction of mixis could help to answer these questions about the timing and mode of mixis induction.

The bioassay described in this paper could also be used to identify the chemical nature of the mixis induction factor. Bioassay-guided fractionations would allow isolation of the active fraction after chromatographic separations. Knowledge of the chemical structure of the mixis induction factor would be of great importance because it could permit elucidation of the biochemical and genetic mechanisms responsible for the life-cycle transition from asexuality to sexuality (Snell 1998).

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