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Chemical induction of mixis in the rotifer *Synchaeta tremula*

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In rotifers of the genus Brachionus, a chemical factor that accumulates during population crowding is necessary and sufficient to trigger sexual reproduction (mixis). In other monogonont rotifer species, field data and some laboratory studies indicate that mixis is density-dependent; however, to date it is unknown whether this reaction is chemically mediated as in Brachionus. Here we provide experimental evidence that mixis in the rotifer Synchaeta tremula is both density-dependent and chemically mediated. S. tremula cultured at high population densities (10 individual mL^{-1}) produced 15.4% mictic offspring, whereas those cultured at low population densities (0.25 individual mL^{-1}) produced only 0.3% mictic offspring. Conditioned water isolated from exponentially growing mass cultures induced significantly higher levels of mixis in the offspring of individually cultured females, when compared with untreated control medium (15.7 versus 1.4% mixis). In S. tremula, the propensity of females to respond to the mixis chemical decreased strongly with age. The highest proportion of mictic offspring (up to 63%) was produced by females of the youngest adult age class (~24–48 h old). Females older than 3 days were virtually unresponsive to the mixis stimulus.

INTRODUCTION

Phenotypic plasticity in the life cycle of zooplankters is often mediated by infochemicals (Larsson and Dodson, 1993). Examples are predator kairomones that induce defensive structures or changes in the timing of development (Tollrian and Harvell, 1999; Lass and Spaak, 2003) or chemicals that suppress growth and reproduction (Folt and Goldman, 1981; Conde-Porcuna, 1998; Burns, 2000; Lürling *et al.*, 2003). The induction of sexual life cycle stages is another important component that can be influenced by chemicals, and this applies for zooplankters with heterogonic life cycles, such as cladocerans or rotifers. In both groups, population crowding can induce the production of sexual stages (Gilbert, 1963, 2004; Kleiven *et al.*, 1992). In rotifers of the genus *Brachionus*, crowding chemicals that accumulate at high population densities are necessary and sufficient to elicit the production of sexual females (Stelzer and Snell, 2003, 2006).

The life cycle of monogonont rotifers involves an alternation between parthenogenetic and sexual reproduction (mixis). During induction of mixis, parthenogenetic (amictic) females produce sexual (mictic) daughters, which then produce either resting eggs or males, depending on whether they have been fertilized or not. Three proximate factors for mixis induction have been identified so far. In several *Asplanchna* species, dietary α-tocopherol induces mixis as well as morphological changes (Gilbert, 1980). In *Notomata*, a change in the photoperiod can induce mixis (Pourriot and Clement, 1981). Finally, in *Brachionus* spp. mixis is induced by a density-dependent chemical (Gilbert, 1963; Stelzer and Snell, 2003, 2006). Although these are the proximate factors that trigger the mixis response, its actual magnitude may depend on other factors as well. For example, the level of mixis is often genotype-dependent, with some clones consistently showing higher or lower levels of mixis (Gilbert, 2003a). Another potentially

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important factor is 'delayed mixis', meaning that females tend to be less responsive to the mixis stimulus for a few generations after hatching from resting eggs (Gilbert, 2002; Schröder and Gilbert, 2004; Serra *et al.*, 2005). Finally, environmental factors such as unfavourable conditions (low food, extreme salinity and toxicants) tend to suppress the response to the mictic stimulus (Snell, 1986).

The adaptive significance of density-dependent mixis in *Brachionus* is well understood. The association of sexual reproduction with high population densities is probably related to the fact that *Brachionus* males cannot locate females from a distance and rely solely on chance encounters (Snell and Garman, 1986). Hence, density-dependent mixis induction increases the probability of male–female encounters and maximizes the production of resting eggs (Serra and King, 1999; Gilbert, 2003a; Serra *et al.*, 2004). Theoretically, these arguments should apply for other monogonont rotifer species as well.

In other monogonont rotifer species, field studies indicate that mixis is often associated with population peaks (Carlin, 1943; Zimmermann, 1974; Schröder, 2001; Virro, 2001). Additionally, a recent experimental study provided direct evidence for density-dependence of mixis in two non-brachionid rotifers (Schröder and Gilbert, 2004). However, so far it is not known whether chemical induction is the mechanism behind this response. In this study, we test the hypothesis that mixis induction in the rotifer *Synchaeta tremula* is both density-dependent and chemically mediated. More specifically, we test (i) whether females show elevated mixis rates if kept at high population densities and (ii) whether individually cultured females induce mixis if kept in conditioned medium (i.e. medium conditioned by dense populations).

METHOD

Synchaeta tremula was isolated in early spring 2005 from a shallow pond close to the Institute of Evolution and Ecology in Münster (North-West Germany). We established a monoclonal culture from one individual stem female. Rotifers were maintained in 0.45-μm filtered pond water (GFF, Whatman) that was mixed 1:1 with Marine Biological Laboratory (MBL) medium (Guillard, 1975). We used *Cryptomonas erosa* as food algae (kindly provided by W. Lampert), which was cultured in pure MBL medium. Food concentrations were 3 mg C L^{-1} (~20 000 cells mL^{-1}). The algal and rotifer cultures were maintained at 16°C and at a photoperiod of light : dark 12:12 h. Stock cultures of algae and rotifers were maintained in sterile Erlenmeyer flasks. In the experiments, rotifers were cultured in polystyrene Petri dishes or in the concavities of tissue culture plates (6-wells or 24-wells).

Pre-cultures

To provide amictic females for the two experiments, we pre-cultured females individually in 20 mL medium in polystyrene Petri dishes. Each time their first offspring hatched (i.e. after ~48 h), the new generation was transferred into fresh medium. Under these conditions, *S. tremula* reproduces exclusively asexually (Timmermeyer, personal observation). After culturing the animals for at least three generations under these conditions, juvenile offspring (age 0–2 h) were collected from different mothers and randomly assigned to the different experimental treatments.

Crowding experiment

The first experiment addressed the question whether population crowding can induce sexuality in *S. tremula*. Experimental females were assigned to two treatments. A high-density treatment contained five replicates and was initiated with each 10 juveniles (taken from the pre-cultures) per 1 mL culture medium (i.e.10 individual mL^{-1}). A low-density treatment contained 10 replicates with five juveniles in 20 mL culture medium (i.e. 0.25 individual mL^{-1}). Because *S. tremula* release their eggs directly into the medium, we had to use a special transfer procedure. Experimental females and culture medium were transferred to new wells/dishes in the morning and in the evening during the 5 consecutive days of the experiment. After the transfers of the females, algae were added from dense cultures to replenish grazed food. The emptied wells/dishes were filled with fresh medium and incubated for another day until all offspring had hatched. Subsequently, offspring were sorted individually into the concavities of 24-well tissue culture plates (1 mL medium), cultured for an additional two days and then typed as mictic or amictic female, depending on the offspring type they produced. For statistical analysis, we pooled data of two randomly chosen pairs of dishes of the low-density treatment, to ensure that our estimates of the mixis rates in both treatments were based on approximately the same number of analysed offspring. We used the Mann–Whitney *U*-test on the percentage of mictic offspring per well/dish as a dependent variable. Differences in the mixis rates among consecutive days of the experiment were analysed by a row-by-column test of independence and the χ^2 statistic (high-density treatment only).

Conditioned water experiment

In the second experiment, we cultured individual females in large volumes (4 mL) and exposed them to conditioned medium from exponentially growing rotifer mass

cultures. Population densities in these mass cultures had reached at least 50 individual mL^{-1} at the time when conditioned medium was prepared. To obtain a measure of the inductive strength of the conditioned medium, we determined mixis rates in the mass cultures by sampling and typing each of 24 newborn females. To prepare conditioned medium, we pooled 60 mL medium from several mass cultures. Rotifers were removed with a 30-μm sieve, and the culture medium was filtered through 0.45-μm glass fibre filters (Whatman GFF). Finally, *Cryptomonas* algae were added from dense cultures. Conditioned medium was freshly prepared during each day of the experiment. The experiment was initiated with 50 juveniles (from the pre-cultures) that were exposed to conditioned medium and 50 juveniles that were exposed to untreated culture medium, which served as a negative control. The experiment was run for 3 days, and offspring of the experimental animals were isolated in the morning and evening of each day using the transfer schedule as described in the first experiment. This resulted in five consecutive samples where eggs had been isolated from the mothers: sample 1 (morning of the second day), sample 2 (evening of the second day), sample 3 (morning of the third day), sample 4 (evening of the third day) and sample 5 (morning of the fourth day). For the analysis of age-specific effects, we condensed these five samples into three age classes: age class 1 (sample 1), age class 2 (samples 2+3) and age class 3 (samples 4+5). The actual process of mixis induction, that is, the decision whether an individual offspring will be mictic or amictic, happens very early in development, most likely during oocyte growth in the mother animal (Gilbert, 2003a). For our samples, this means that all eggs, which are found on the bottom of the wells, were already determined with respect to their fate (mictic or amictic offspring). Induction probably happened several hours before, during the production of these eggs. Thus, our age classes 1–3 correspond closely to the actual timing of mixis induction on the experimental days 1–3, respectively. To test for differences in mixis among the different age classes, we analysed the frequencies of mictic and amictic daughters with a row-by-column test of independence and the χ^2 statistic (conditioned water treatment only).

RESULTS

The results of our first experiment showed that population crowding can induce high levels of mixis in the experimental females. Four replicates in the high-density treatment (10 individual mL^{-1}) showed mixis levels ranging between 15.7 and 28.3% (Fig. 1). Inexplicably, the fifth replicate showed no mictic offspring at all. Most low-density treatments (0.25 individual mL^{-1}) resulted in amictic offspring only. Just in one replicate, one mictic offspring was found (replicate 'K', see Fig. 1). On average, our crowding treatment induced 15.4% mictic offspring, which was statistically significant (Mann–Whitney *U*-test: $P < 0.05$, $n = 5$). A closer look at the temporal pattern of mixis in the high-density treatment showed that mixis levels differed considerably during consecutive days of the experiment (Fig. 2). The highest value of 27.7% was reached on the third day, followed by 14% on the fourth day. In contrast, on the second and on the fifth day only a very low percentage of mictic offspring was produced (<2%). This temporal heterogeneity of mixis rates was statistically significant (row-by-column test, $\chi^2 = 38.02$, $df = 2$, $P < 0.001$).

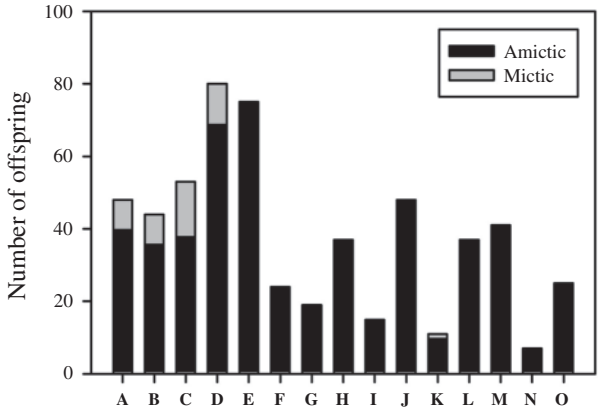

Fig. 1. Effect of population crowding on the induction of mixis in *Synchaeta tremula*. A–E, high-density treatments (10 females in 1 mL medium); F–O, low-density treatments (5 females in 20 mL).

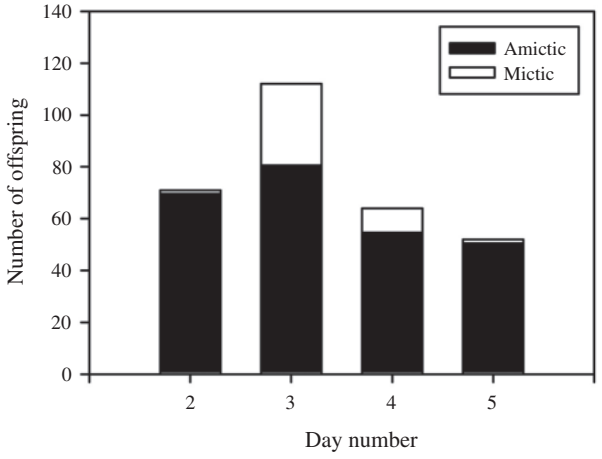

Fig. 2. Development of mixis during the crowding experiment. Only the high-density treatment is displayed.

In the second experiment, conditioned medium was prepared from mass cultures that grew exponentially and showed high levels of mixis induction (Table I). Population density ranged between 50 and 200 individual mL^{-1} during the harvest of conditioned medium. Experimental females treated with conditioned medium produced on average 15.7% mictic offspring, in contrast to 1.4% mictic offspring in the negative control. This difference was highly significant (Mann–Whitney *U*-test: $P < 0.001$, $n = 42–45$). The responses of individual females were very variable: some females produced up to 45% mictic offspring, whereas others produced no mictic offspring at all (Fig. 3).

An alternative way to analyse mixis levels is to score the experimental females according to two categories: those that produce at least one mictic offspring during the experimental period versus those that produce no mictic offspring at all. In this case, the proportion of females producing at least one mictic offspring is significantly higher if they are cultured in conditioned medium (74 versus 14%; Fisher's exact test: $P < 0.001$). Overall, this shows that the treatment with conditioned medium had two effects: (i) conditioned medium increases the proportion of experimental females that produce any mictic offspring at all and (ii) conditioned medium increases the average percentage of mictic offspring of the experimental females. We observed no significant difference in the reproductive rates of females treated with conditioned versus control medium (9.1 versus 8.7 offspring per female; $P = 0.307$, two-tailed Student's *t*-test).

Similar to the first experiment, we observed temporal variation of mixis induction. In Table II, we have listed the levels of mixis induction in three age classes of the experimental females. These age classes should roughly correspond to the three successive treatments with conditioned water (see *Method* for definition of the age classes). The mixis levels in the mass cultures from which the conditioned medium was prepared showed only little variation (40.5–46.2%). In contrast, mixis induction in the experimental females showed a steep decrease with the age of the experimental females, ranging from 63.3% in age class 1 (~24 h old females) to 0.5% in age class 3 (~72 h old females). This effect of age class on mixis was highly significant (row-by-column test, $\chi^2 = 114$, $df = 2$, $P < 0.001$).

DISCUSSION

To our knowledge, this is the first description of *S. tremula* in laboratory culture. In our mass cultures, we observed an unusual behaviour. Females, as well as males, constantly secreted a sticky fibre from a pore near the cloaca. After extended periods in the same vessel, the animals formed large 'web'-like structures. Freshly laid eggs

Table I: Mass cultures used for the production of conditioned medium

Culture	Day	Individual mL^{-1}	Cond	% Mixis	Total	f	m
A	0	0.2		—			
	4	2.6		—			
	6	48.6	1	33.3	24	5	3
	7	103	2	50.0	22	11	0
	8	258		50.0	22	11	0
	9	—		—			
B	0	0.2		—			
	4	1.5		—			
	6	48.2	1	47.8	23	7	4
	7	87		—			
	8	186	3	35.7	14	5	0
	9	165		—			
C	0	0.1		—			
	4	1.6		—			
	6	—		—			
	7	70	2	37.5	24	9	0
	8	103	3	56.8	37	19	2
	9	130		—			

Cond, experimental day, on which conditioned medium was prepared; Day, days since inoculation of the mass culture; f, fertilized mictic females; m, unfertilized mictic females (male producing); total, total number of juveniles sampled (for estimation of mixis rate).

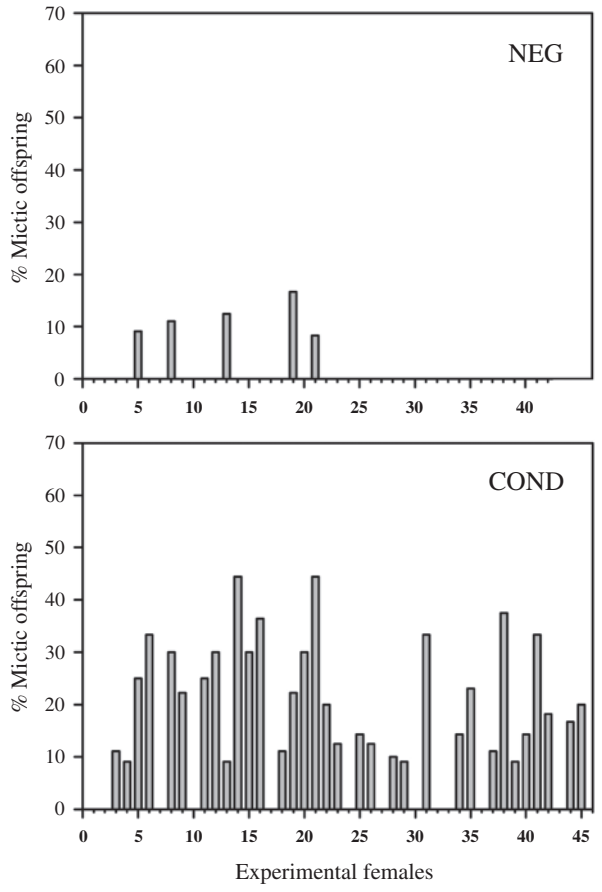

Fig. 3. Response of individual females to conditioned medium (COND) and untreated control medium (NEG). COND was 0.45-μm filtered medium from exponentially growing mass cultures of 50–200 individual mL^{-1}, which had mixis levels of 40–45%.

Table II: Influence of female age on mixis induction in the 'conditioned water experiment'

	Age class	Mixis (%)	*n*	MC-mixis (%)
COND	1	63.3	30	40.5
	2	27.3	172	43.7
	3	0.5	217	46.2
NEG	1	0.0	31	—
	2	2.1	184	—
	3	1.2	155	—

Age class, roughly correspond to the successive exposures to conditioned medium in the experiment (see text for details); COND, conditioned medium; MC-mixis, mixis in the mass culture from which the conditioned water was prepared (average of two cultures); *n*, number of offspring examined; NEG, untreated control medium.

usually got stuck to these webs. Also, offspring hatching from the eggs often stayed connected to the webs of their stem mother, forming colony-like structures. Although the webs themselves may be a culture artefact, the production of the fibre could indeed be of significance in the natural habitat: *S. tremula* is more common in the littoral zone than in the pelagic zone (Pontin, 1978); thus, the fibre may allow these animals to forage in the water column while it prevents them from drifting away from the shore.

Our results clearly demonstrate that mixis in *S. tremula* is induced by a density-dependent chemical factor. Mixis was significantly induced when animals were kept at high- versus low population density, as well as when individuals were cultured in medium conditioned by a mass culture versus control medium. Thus our study provides the first clear example that chemical induction of mixis is not confined to the genus *Brachionus*. The number of offspring per female was not affected by the conditioned medium in our *S. tremula* clone. In other zooplankton, conditioned water sometimes reduces the reproductive rates (Burns, 1995; Lürling *et al.*, 2003).

Age of the experimental females had a significant influence on their response to the mixis factor. This was most obvious in the 'conditioned medium experiment'. The highest proportion of mictic daughters was induced within the first 24 h of adulthood, and mixis induction decreased sharply afterwards. This decrease was likely because of changes in the females' responsiveness to the mixis signal rather than because of a decrease in the signal intensity, because the mixis levels in our mass cultures suggest that the conditioned medium had a constant inductive strength throughout the experiment. A similar pattern of age-related changes in mixis induction was apparent in the crowding experiment, although in this case the strong peak of mixis in the first 24 h of adulthood was absent. However, in this experiment no conditioned medium was used—the mixis chemical must have been secreted by the experimental animals themselves. It is thus possible that the chemical was not present in sufficient concentrations during the first 2 days of the crowding experiment. Age-related changes in mixis propensity have been reported by other authors as well. No uniform picture has emerged so far: mixis propensity has been found to increase or decrease with age or in some species to peak in the middle of the reproductive period (summarized in J. J. Gilbert and T. Schröder, submitted for publication).

One could argue that there is quantitative inconsistency in our data: conditioned medium, which induced 40–45% mixis in the mass cultures, induced only 15% mictic offspring in the experimental animals. As already mentioned, the age classes 1 and 2 (~24–48 h) were the most responsive ones to the mixis stimulus (up to 63% mixis induction), whereas age class 3 hardly showed any propensity to produce mictic offspring. The calculated value of 15%

mictic offspring in the conditioned water treatment refers to the average across *all* offspring produced by the age classes 1–3. In an exponentially growing population, the age distribution is typically skewed, with an excess of young age classes. This may indeed explain the higher mixis levels in our (exponentially growing) mass cultures. A similar discrepancy, between animals treated with conditioned medium versus the corresponding mass culture, was previously described in *Brachionus plicatilis* (Stelzer and Snell, 2003). In this study, mixis rates in the experimental animals reached only 51% of those in the mass culture. Several alternative explanations were discussed by the authors, for example, instability of the mixis chemical or adsorption onto filter membranes during preparation of the conditioned medium.

Mixis levels never reached 100% in our experiments. However, this does not challenge the importance of chemical induction. Indeed, mixis induction well below 100% is expected from theoretical considerations (Serra and King, 1999). This can be explained by a bet-hedging strategy that allows clonal populations to produce starvation-resistant resting eggs while continuing with asexual population growth, in case the conditions stay favourable. Some studies have shown that there is genetic variation for such mixis propensity, some clones consistently showing low/high mixis induction (Gilbert, 2003a).

Mixis induction by density-dependent chemicals may more be widespread than it is currently appreciated. Recently, Schröder and Gilbert (Schröder and Gilbert, 2004) have demonstrated density-dependent mixis in two non-brachionid species, *Rhinoglena frontalis* and *Epiphanes senta*. In fact, even the α-tocopherol-induced mixis response in *Asplanchna* spp. can be significantly enhanced by crowding (Birky, 1969). It is thus possible that these responses were also chemically mediated. Chemical induction of mixis raises several evolutionary and ecological questions. One question would be whether mixis chemicals have evolved independently or whether they are an ancient trait in monogonont rotifers that became subsequently modified during evolution. Some of these modifications could have merely changed the signal/receptor structure, whereas others could have combined chemical induction with other modes of mixis induction. From an ecological perspective, it would be interesting to know how co-occurring rotifer species avoid interference in their chemical communication systems. Many rotifer species in freshwater habitats show temporal and spatial overlap due to niche partitioning and hence may be exposed to each other's chemicals. Experimental studies on species specificity of mixis in *Brachionus* gave mixed results, some showing species specificity (Gilbert, 2003b), whereas others demonstrated cross-induction among species (Stelzer and Snell, 2006). Thus, a more detailed characterization of the chemical mixis signal (Snell *et al.*, 2006) may help to resolve such discrepancies and provide further insights into the evolution and diversification of the mixis signal.

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