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Obligate asex in a rotifer and the role of sexual signals

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Abstract

Transitions to asexuality have occurred in many animals and plants, yet the biological mechanisms causing such transitions have often remained unclear. Cyclical parthenogens, such as cladocerans, rotifers or aphids often give rise to obligate asexual lineages. In many rotifers, chemical signals that accumulate during population crowding trigger the induction of sexual stages. In this study, I tested two hypotheses on the origin of obligate parthenogenesis in the rotifer *Brachionus calyciflorus*: (i) that obligate parthenogens have lost the responsiveness to the sexual signal; and (ii) that obligate parthenogens have lost the ability to produce the sexual signal. Pairwise cross-induction assays among three obligate parthenogenetic strains and two cyclically parthenogenetic (sexual) strains were used to test these hypotheses. I found that obligate parthenogens can induce sexual reproduction in sexual strains, but not vice versa. This demonstrates that obligate parthenogens do still produce the sexual signal, but have lost responsiveness to that signal.

Introduction

Although asexual lineages have emerged in almost all major animal and plant groups, most of them are doomed to early extinction (Maynard Smith, 1978; Bell, 1982). The short evolutionary life of asexual lineages has been explained by a lack of recombination, which renders their genomes prone to the accumulation of deleterious mutations and/or less efficient for the fixation of adaptive mutations (e.g. reviews by Hurst & Peck, 1996; Rice, 2002). In addition, asexual lineages possibly lack the ability to generate sufficient genetic variation in short term to counter changing selection pressures, such as those posed by rapidly coevolving parasites. Despite the short evolutionary life of most asexual lineages, a dynamic equilibrium between sexual and asexual forms can result, e.g. if old asexual lineages are continuously replaced by new ones (Butlin *et al.*, 1999; Simon *et al.*, 2003; Paland *et al.*, 2005). Such repeated origins of asexuality have been documented in many animals (Simon *et al.*, 2003) and plants (Sharbel & Mitchell-Olds, 2001; van Dijk & Vijverberg, 2004). Understanding the proximate causes for the origin of new asexual lineages remains a major challenge, as such causes determine the frequency of asexual transitions as well as the levels of genetic diversity within asexual populations (Simon *et al.*, 2003).

Repeated origins of obligate asexuality have been described in cyclical parthenogens, i.e. organisms that are capable of both sexual and parthenogenetic reproduction (Delmotte *et al.*, 2001; Paland *et al.*, 2005). A cyclical parthenogenetic life cycle enables organisms to cope with periodical changes in the environmental conditions, such as those observed during an annual cycle. During favourable periods, asexual reproduction allows cyclical parthenogens to quickly exploit resources and to build up large populations. Once environmental conditions indicate future deterioration, sexual stages are induced, and these often culminate in the production of diapausing stages (e.g. resting eggs). Interestingly, the proximate stimuli that induce sex often serve as direct or indirect cues of deteriorating future conditions, e.g. population crowding, declining food concentrations or a reduction in day length (Kleiven *et al.*, 1992; Stelzer & Snell, 2003). One hypothesis for an origin of new parthenogenetic lineages is that these fail to respond to the sex-inducing stimuli (Simon *et al.*, 2003). In this study, I tested this hypothesis using sexual (cyclical

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parthenogenesis) and asexual (obligate parthenogenesis) strains of the rotifer *Brachionus calyciflorus* Pallas.

Monogonont rotifers are small invertebrate metazoans of worldwide distribution represented by approximately 1500 described species and found mainly in freshwater habitats (Wallace *et al.*, 2006). Their close relatives, the bdelloid rotifers are one of the few asexual lineages for which there is substantial evidence for ancient asexuality (Mark Welch & Meselson, 2000; Mark Welch *et al.*, 2004). The monogonont life cycle involves cyclical parthenogenesis, an alternation of phases with asexual reproduction (amixis) and sexual reproduction (mixis). The asexual phase is characterized by rapid clonal propagation: amictic females produce diploid eggs by ameiotic parthenogenesis. The sexual phase is initiated when amictic females produce mictic female offspring. Mictic females produce haploid eggs that develop into haploid males if unfertilized. Fertilized mictic eggs, however, develop into diapausing eggs that can undergo extended periods of dormancy. Amictic females hatching from diapausing eggs initiate another phase of parthenogenetic reproduction, and the cycle starts again.

The induction of mixis is triggered by a chemical that is produced by the rotifers themselves and accumulates during population crowding, commonly at rotifer densities above 0.1 individuals per millilitre (Gilbert, 1963; Carmona *et al.*, 1993; Stelzer & Snell, 2003, 2006; Timmermeyer & Stelzer, 2006). This chemical cue is necessary and sufficient for the induction of mixis, unless the physiological state of the animals is disturbed by extreme environmental conditions (Snell, 1986). If females are exposed to the mixis chemical, they produce a certain fraction of mictic daughters (Gilbert, 2003a; Stelzer & Snell, 2006). This fraction is often clone-specific and typically ranges between 5% and 50% (Gilbert, 2003a). A recent study has shown that the mixis chemical of *Brachionus plicatilis* is a water-soluble protein (Snell *et al.*, 2006).

There have been several reports of obligate parthenogenetic strains in *Brachionus* sp. (e.g. Buchner, 1987; Bennett & Boraas, 1989; Fussmann *et al.*, 2003). Given the mode of chemical induction of mixis, there are two main possibilities by which a transition to obligate parthenogenesis can evolve. First, obligate parthenogens could have become insensitive to the mixis-inducing chemical. This could be caused, for instance, by a mutation in the receptor of the mixis chemical or at other sites in the downstream signalling cascade. Obligate parthenogens should have a demographic advantage and spread in the population, because they would produce amictic (i.e. nondiapausing) offspring even when population densities are high. Second, obligate parthenogens may have abandoned the production of the sex-inducing chemical. This by itself might not grant a demographic advantage, as other clones that still produce the mixis chemical might be present in the population. However, if the production the sexual signal were energetically costly, obligate parthenogens could use the spared resources for the production of more offspring. In this study, I used cross-induction assays with conditioned media, produced by cyclically parthenogenetic (hereafter sexual) and obligately parthenogenetic (hereafter asexual) strains of the rotifer *B. calyciflorus*. I found that mixis was induced in sexual strains when treated with conditioned medium of asexual strains, but not vice versa. This strongly suggests that asexuals do still produce the chemical signal that induces sex, but have lost responsiveness to that signal.

Methods

Study species

Five different strains of the rotifer *B. calyciflorus* were used in the experiments. Three strains of *B. calyciflorus* (TX, FL and GA) were supplied by J.J. Gilbert (Dartmouth College, Hanover, NH, USA), one strain (NY) was supplied by N.G. Hairston (Cornell University, Ithaca, NY, USA) and one strain (EG) was isolated in autumn 2006 from lake Egelsee, a small lake in the vicinity of the Institute for Limnology (Mondsee, Austria). Four of these strains were used in earlier studies (e.g. NY in Fussmann *et al.*, 2003; TX, FL and GA in Gilbert & Walsh, 2005). Three of the strains were obligate parthenogens (FL, GA and NY), i.e. they produced only amictic female offspring, irrespective of population density. The other two strains (EG and TX) were cyclical parthenogens, which produced a certain fraction of mictic females, males and sexual eggs. Sexual eggs are diapausing and take several weeks to develop (Hagiwara & Hino, 1989), whereas asexual eggs develop within about 8 h at 25 °C (Bennett & Boraas, 1989). Thus, in laboratory, where cultures are frequently inoculated or continuously diluted (such as in chemostats, see below), the sexual strains are also propagated clonally. During the course of this study, I did not observe any reduction in the investment into mictic offspring in the sexual strains (EG and TX). Rotifers were fed the alga *Chlamydomonas reinhardii* Dangeard (Strain: SAG11-32b, Sammlung für Algenkulturen, Göttingen, Germany). Both, rotifers and algae, were cultured in COMBO, an artificial freshwater medium enhanced with trace elements and vitamins (Kilham *et al.*, 1998).

Chemostat culturing

During the experiments rotifer populations were cultured in a two-stage chemostat system, in which they were kept in a dynamic equilibrium due to constant inflow of fresh food suspension and constant removal of animals and their waste products. The first stage of the chemostat system consisted of a large-volume algal chemostat (2100 mL), in which the food alga *C. reinhardii* was cultured. The algal chemostat was continuously

illuminated by two daylight fluorescent bulbs (irradiation intensity: ~200 μE s^{-1} m^{-2}) and operated at a dilution rate of 1.1 day^{-1}. The inflowing medium was 0.2-μm filtered COMBO medium, which was provided in a 20-L bottle that was exchanged once per week. Under these conditions, the algal chemostat entered a steady state with a relatively constant algal biovolume of 1.2×10^8 fL mL^{-1} (femto liter) (~750 000 cells mL^{-1}). The second stage of the chemostat system consisted of 12 rotifer chemostats (each 380 mL volume). Food suspension was harvested from the algal chemostat with a peristaltic pump, mixed 1 : 1 with sterile COMBO medium in a small reactor vessel and the automatically distributed into the 12 rotifer chemostats using a 12-channel peristaltic pump. The rotifer chemostats were kept in a water bath at 24 ± 0.2 °C and were illuminated continuously at a low light intensity (~5 μE s^{-1} m^{-2}). The outflow of the rotifer chemostats was collected in 1-L glass bottles, which were replaced every day. All components of the chemostat system (i.e. glass vessels, lids and tubing) were autoclaved and assembled in a stream of sterile air (provided by gas burner). Chemostats were continuously bubbled with filtered air (0.2-μm PTFE filters). For each of the five rotifer clones there were two to three replicate chemostats (GA, FL and EG clones: two chemostats each; TX and NY clones: three chemostats each). In the beginning of the experiment, the rotifer chemostats were inoculated with 170 females of the respective clone. The dilution rate was set to 0.87 day^{-1}. This means that each rotifer population had to multiply at least by a factor of 2.38 day^{-1} to remain at a constant population density.

Chemostat sampling

The 12 chemostats were sampled daily to determine the population density of the rotifers. Samples were taken by withdrawing culture suspension directly into a 20-mL sterile test tube using vacuum; this test tube was exchanged with every sampling. Rotifers were counted alive or fixed with a drop of Lugol's solution for later counts using inverted microscopy. To facilitate comparisons of the population dynamics between different clones, I calculated the exponential growth rate r for the initial time periods when the populations were still growing and the average population density, for the time periods when the populations were not growing any more (i.e. an approximation of the 'carrying capacity'). The exponential growth rate was calculated as the slope of the regression line fitted trough the natural logarithm of abundance vs. time for each replicate. For these calculations, I included all measurements up to the time point at which population size stopped to increase. All remaining time points of each replicate were used to calculate the mean population densities (carrying capacity).

On several occasions (days 5, 7, 10, 11, 12, 16 and 17), mixis rates in the chemostat populations were estimated from a sample of 48 young, non-egg-bearing females that were isolated individually in 48-well polystyrene plates, each well filled with 500 μL of food suspension. After 2 days of incubation, the reproductive type of the females was determined. Females were classified as mictic if they produced males or resting eggs or amictic if they produced females.

Mixis bioassay

A mixis bioassay similar to that described in Stelzer & Snell (2006) was used to test for cross-induction between asexual and sexual clones. In this bioassay, newborn females were presented with culture medium that had been conditioned by a dense population of another rotifer clone and the level of mixis induction was measured (per cent sexual daughters). Positive and negative controls were made with water conditioned by the focal clone itself and with unconditioned medium respectively. Conditioned medium was prepared fresh each day from the overflow of the rotifer chemostats (collected within a 24-h period). Rotifers were removed with a 30-μm sieve. After centrifugation for 30 min at 12 000 × g the supernatant was carefully decanted and filtered through Whatman GF/C glassfibre filters (Whatman, Maidstone, UK). For the negative controls, fresh COMBO medium was used.

In this study, five different clones were examined. Hence, there were 30 treatments, which included the 5 × 5 combinations of each focal clone and conditioned medium, plus five negative controls (one for each focal clone). For each treatment, eight to 12 replicate females were used, and on average five offspring of each replicate female were examined for their reproductive type (mictic or amictic). To provide amictic females for the experiments, 25 females of each clone were cultured at a low population density for at least three generations. Briefly, newborn females were introduced singly into 20 mL of food suspension in a polystyrene Petri dish. After 2 days, the first newborns hatched and one of them was used to start a new subculture. At the start of the bioassay, focal animals (juveniles hatched within a 8-h period) were introduced into a new Petri dish containing a mixture of 10 mL of conditioned medium and 10 mL of fresh algal suspension (final algal concentration: ~40 000 cells mL^{-1}). Everyday, focal animals were transferred to new dishes with freshly prepared conditioned medium. Any offspring that hatched within this 24-h period were isolated and individually transferred into the wells of a 48-well polystyrene plate, each well filled with 0.5 mL of food suspension. After 2 days, the reproductive type of these females was determined in the same way as in the bioassay used for the chemostat populations (see above). The mixis bioassay lasted from day 10 to day

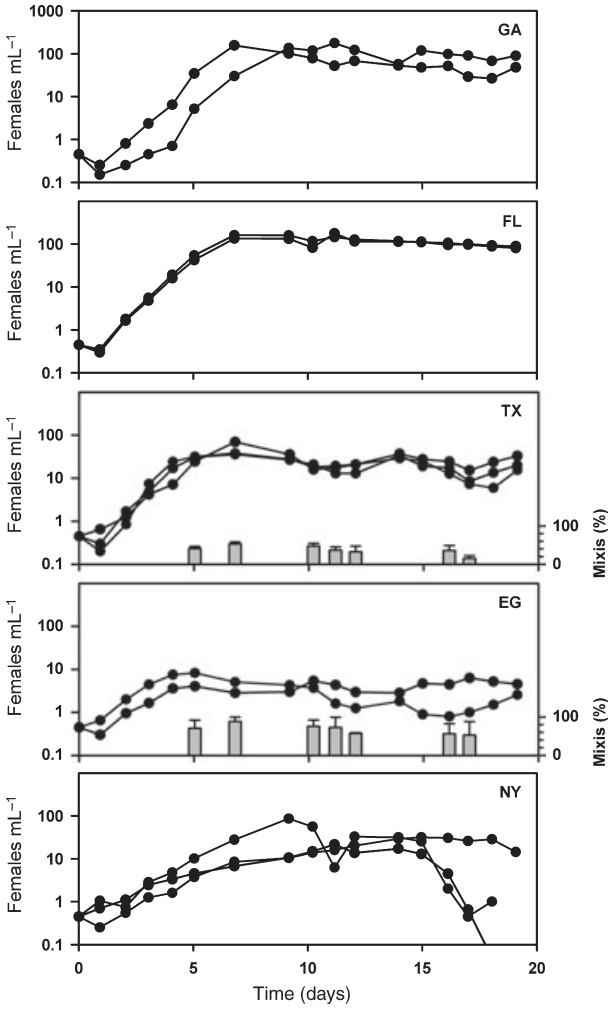

Fig. 1 Population dynamics (lines and filled circles) and mixis rates (grey bars) of the different rotifer strains in the chemostats. Note the logarithmic scaling on the left *Y*-axis. There were two replicate chemostats for the GA, FL and EG strains, and three replicate chemostats for the TX and NY strains. Mixis rates (i.e. per cent sexual females) for the sexual TX and EG strains are shown in the right *Y*-axis. Mixis rates were determined only on days 5, 7, 10, 11, 12, 16 and 17. The obligate parthenogenetic strains (GA, FL and NY) produced no mictic offspring at all.

14 of the chemostat trial (see Fig. 1). The data were analysed using the nonparametric Kruskal–Wallis test, corrected for ties, to compare the percentages of mictic offspring for each clone treated with different conditioned media. In those cases where the Kruskal–Wallis test yielded a significant value, pairwise comparisons were performed with the Conover–Inman test to identify homogeneous groups of treatments (Software: StatsDirect Ltd., Altrincham, UK). Homogeneous groups were defined as the treatments that were not significantly different among each other, but differed significantly from other treatments ($P < 0.05$).

Results

Chemostat cultures

All rotifer populations in the chemostats showed an initial increase in population density followed by equilibria with more or less constant population density (Fig. 1). The two asexual clones, FL and GA, showed the highest population growth rates of all clones (Table 1). Note that Table 1 gives the population growth rates of the chemostat populations, which were subject to a constant dilution rate of 0.87 day^{-1}. Hence, the actual maximum growth rates of the FL and GA clone were 1.88 and 1.92 day^{-1}, respectively, under the experimental conditions. In other words, these two clones have the capacity to double in population size approximately every 8.7 h. The two obligate asexual clones, FL and GA, also reached the highest mean population densities (Table 1). Interestingly, the EG clone had the lowest mean population density of all clones (Table 1), which was likely caused by its tendency to induce the highest levels of mixis. The NY clone behaved somewhat differently from the other four clones. Its initial population growth was much slower than that of the other clones. In fact, it was still growing monotonically after day 7, when all the other clones had already reached their carrying capacities (Fig. 1). Towards the end of the experiment the NY clone was even washed out in two replicate chemostats, which indicates that this clone was close to its limits of growth under the set dilution rate.

Mixis bioassay

In the mixis assay, I tested whether conditioned medium from each of the five clones (GA, FL, TX, EG and NY) would induce mixis in others. Females of the asexual clones (NY, FL and GA) did not produce any sexual offspring at all during the experiment (Table 2). By contrast, the sexual clones that were treated with conditioned media produced high proportions of mictic offspring (Table 2). These levels of mixis were significantly higher than the negative control in most cases

Table 1 Population dynamical parameters of the different clones of *Brachionus calyciflorus*.

Clone	Exponential growth rate	Carrying capacity (day^{-1}) (individuals mL^{-1})
GA	1.01 (±0.16)	79.7 (±34.4)
FL	1.05 (±0.01)	113.5 (±2.6)
TX	0.87 (±0.06)	20.7 (±3.9)
EG	0.63 (±0.01)	3.2 (±1.4)
NY	0.41 (±0.16)	23.7 (±8.4)

Exponential growth rate during exponential growth after inoculation of the chemostats. Carrying capacity (i.e. mean population density), once the populations ceased to grow monotonically. Means and standard deviations (n = 2–3).

Table 2 Mixis induction among different clones of *Brachionus calyciflorus*.

	Conditioned medium					
Induced clone	GA	FL	TX	EG	NY	NEG
GA	0	0	0	0	0	0
FL	0	0	0	0	0	0
TX	21	5	12	30	27	3
EG	55	40	40	43	37	4
NY	0	0	0	0	0	0

Per cent mixis in offspring of the experimental animals. NEG, untreated culture medium. Asexual strains (GA, FL and NY); sexual strains (TX and EG).

Table 3 Statistical analysis of mixis induction in the TX and EG clones.

Induced clone	Kruskal–Wallis test		Groups after Conover–Inman test (conditioned media)
	H	*P*	
TX	17.64	0.0034	(NEG, FL, (TX), GA, NY, EG)
EG	13.04	0.023	(NEG), (NY, FL, TX, EG, FL)

Results of the Kruskal–Wallis test and *post hoc* Conover–Inman test on mixis induction of conditioned media from different clones (EG, FL, GA, NY and TX) and untreated culture medium (NEG). Groups of conditioned media that did not differ significantly ($P > 0.05$) from each other are clustered in brackets.

(Kruskal–Wallis test, $P < 0.05$, Table 3). The EG clone induced high levels of mixis (37–55%) with any of the conditioned media and there were no significant differences among them (Table 3). In the TX clone, mixis induction was more variable. The treatments with FL- and TX-conditioned medium resulted in rather low proportions of mictic offspring (5% and 17%), which were not significantly different from the negative control (3% mictic offspring). However, the other treatments (NY, GA and EG) induced mixis at levels that were significantly higher than the negative control. Overall, there were several cases of cross-induction, most notably those cases where medium conditioned by the asexual clones (NY, GA and FL) significantly induced mixis in the sexual clones TX and EG (Table 3).

Discussion

Obligate parthenogenesis in *B. calyciflorus* seems to be stably inherited; there was no indication of reversal to the cyclically parthenogenetic life style in any of the asexual clones. The NY, FL and GA clones produced no sexual offspring at all during the experiment, and neither did for several months afterwards (C.P. Stelzer, unpublished observation). During the experiment, the offspring of about 500 females of each clone were screened. In addition, daily light microscopical examination of the chemostat populations did not reveal any signs of sexual reproduction, such as the presence of mictic females, males or diapausing eggs. By contrast, the two sexual clones (EG and TX) continuously produced mictic females, often at rates of 50% and higher.

The differences in mixis among the five rotifer clones are probably due to genetic differences and not due to differences in the environment. Several alternatives can be ruled out: first, direct environmental effects are unlikely, as the culture conditions of all five clones were basically identical: all rotifer cultures were fed from the same algal culture and were exposed to the same dilution rate, temperature and light conditions. Secondly, maternal effects can be ruled out, as the experiment lasted 3 weeks in total (~10 asexual generations), which greatly exceeds the time period such effects usually persist (Bernardo, 1996). Thirdly, differences in clonal age, i.e. the number of elapsed generations since the clones hatched from resting eggs, are not likely to be responsible for the differences in sex induction among the studied clones. Gilbert (2002) described a transgenerational effect by which rotifers remain relatively insensitive to the sexual signal for the first 12 generations after hatching from the resting egg. At the time of the experiments, all clones were older than 2 months since hatching, i.e. their clonal age was much larger than 12 generations. In fact, the youngest clone (EG clone, hatched in autumn 2006) showed the highest levels of mixis induction (up to 96% sexual offspring). The differences in sexual reproduction therefore seem to be an intrinsic property of each clone and are not caused by the environment.

The mixis assay provided strong evidence that asexual clones do still produce the sexual signal. Both sexual clones (TX and EG) induced mixis upon exposure to conditioned media of asexual clones (Table 2). There was one exception. The TX clone did not induce significantly when exposed to medium conditioned by the FL clone. However, the TX clone did overall induce lower levels of mixis than the EG clone. In fact, mixis induction with medium conditioned by the TX clone itself was slightly elevated, but not significantly higher than the negative control, which could have been caused by the low sample size. However, the fact that the TX clone did significantly induce mixis with all the other conditioned media was an evidence for cross-induction from asexual clones towards the TX clone. By contrast, the asexual clones (NY, FL and GA) never produced any sexual offspring, not even when treated with conditioned medium from the sexual clones. This suggests that they have lost their responsiveness to the signal that induces sexual reproduction.

An alternative explanation for why asexual clones failed to respond to the sexual clones might be a structural divergence of the mixis chemical between clones (Gilbert, 2003b; Stelzer & Snell, 2006). Such divergence could have happened during speciation and

would result in sexual isolation between diverged lineages (Gilbert, 2003b). It is true that some of the clones of *B. calyciflorus* represent separate lineages and may even belong to different members of a cryptic species complex (Gilbert & Walsh, 2005). Even provided there was divergence of the mixis signal, one would, however, expect sexual isolation in both directions: sexuals should be unable to induce asexuals and vice versa. This was clearly not the case, as asexual clones induced mixis in the sexual clones in most treatments. Clonal differences in the structure of the mixis chemical are therefore not a likely explanation for why asexual clones did not respond to the conditioned media of the sexual clones.

Another alternative explanation is that obligate asexual strains of *Brachionus* use a different cue for induction of sex (e.g. day length). This explanation is very unlikely, as each of the asexual strains evidently did respond to population crowding in the past. For instance, data on the NY strain is published in the study of Fussmann (2003). During the first weeks of that study, the NY strain was inducing mixis upon population crowding, as there was a significant positive relationship between population density and the proportion of mictic females (Fussmann *et al.*, 2003). For the other two strains, FL and GA, similar data exist, demonstrating that these strains also did induce mixis at high population densities in the past (Gilbert, 2003b).

Could the obligate asexual strains have merely evolved an increased threshold for mixis induction? This scenario is unlikely for two reasons. First, the population densities of the FL and GA strains in the chemostats were very high (79 and 113 individuals per millilitre, Table 1). Such densities are at the upper end of what rotifers will ever encounter in nature (Wallace *et al.*, 2006). Hence, even if the FL and GA strains would have evolved higher thresholds for mixis induction, e.g. in the range of several hundreds or thousands of individuals per millilitre, this would be equivalent to nonresponsiveness in ecological terms. Secondly, there seems to be a fundamental difference between obligate asexuals vs. sexuals in their response towards unconditioned medium (which should be a good approximation to a 'below-threshold' mixis signal): whereas the sexual strains sometimes produced a small, yet statistically insignificant number of sexual offspring (Tables 2 and 3), obligate asexuals never produced any sexual offspring at all (Table 2). This suggests that obligate asexuals are really asexual in a strict sense.

To sum up, the most plausible explanation for the origin of asexuality in *B. calyciflorus* is that obligate parthenogens have permanently lost the ability to respond to the mixis chemical. Interestingly, this loss seems to have happened independently in all the three asexual strains of this study (NY, FL and GA), as each derived from a different sexual ancestor. As there have been many other reports on the loss of sexual reproduction in *Brachionus* (e.g. Boraas, 1983; Bennett & Boraas, 1989; Fussmann *et al.*, 2003), heritable variation in the responsiveness to the sexual signal may not be uncommon. Interestingly, this variation may not necessarily rest on high genetic variation, such as in an outbred population, as several transitions to asexuality have been observed in cultures that were founded by one individual female (e.g. Bennett & Boraas, 1989; FL and GA clone of this study). Furthermore, it has recently been shown that several strains of *B. calyciflorus* can exhibit significant intraclonal variation in the propensity for sexual reproduction (Gilbert & Schröder, 2007). The mechanism behind such variation, however, still remains to be determined.

Two further important questions remain. First, is obligate parthenogenesis in monogonont rotifers of any significance in natural populations? A widely accepted model predicts intermediate rates of mixis to be optimal for long-term survival of populations, because there is a trade-off between asexual reproduction – which increases competitive ability and allows larger population sizes, and sexual reproduction – which contributes to population survival through harsh periods by diapausing resting eggs (Serra & King, 1999). However, Fussmann *et al.* (2003) pointed out that there should be periodic selection for obligate parthenogenesis during the growth season, because sexual offspring and diapausing eggs are reproductive dead ends in the sense that they do not contribute to the 'real-time' population dynamics. Overall, stable habitats may select for asexual reproduction and obligate parthenogens should be observed more frequently in such habitats. Unfortunately, to date, there are no studies that quantified the proportion of obligate asexual clones in natural populations of monogononts. Secondly, what are the molecular mechanisms involved in obligate parthenogenesis? This study suggests that such mechanisms should involve changes in the receptor molecule and/or changes in the downstream signalling pathway. A detailed comparison of the molecular characteristics of obligate parthenogens vs. their sexual ancestors would be a promising approach to explore the molecular mechanisms of loss of sex in monogononts.

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