Marine Biology (2006)
DOI 10.1007/s00227-006-0251-2

RESEARCH ARTICLE

Terry W. Snell · Julia Kubanek · William Carter
Audra B. Payne · Jerry Kim · Melissa K. Hicks
Claus-Peter Stelzer

A protein signal triggers sexual reproduction in *Brachionus plicatilis* (Rotifera)

Received: 13 October 2005 / Accepted: 3 January 2006

Abstract The defining feature of the life cycle in monogonont rotifers such as *Brachionus plicatilis* (Muller) is alternation of asexual and sexual reproduction (mixis). Why sex is maintained in such life cycles is an important unsolved evolutionary question and one especially amenable to experimental analysis. Mixis is induced by a chemical signal produced by the rotifers which accumulates to threshold levels at high population densities. The chemical features of this signal were characterized using size exclusion, enzymatic degradation, protease protection assays, selective binding to anion ion exchange and C3 reversed phase HPLC columns, and the sequence of 17 N-terminal amino acids. These studies were carried out over two years beginning in 2003 using *B. plicatilis* Russian strain. When rotifer-conditioned medium was treated with proteinase K, its mixis-inducing ability was reduced by 70%. Proteinase K was added to medium auto-conditioned by 1 female ml^{-1} where typically 17% of daughters became mictic and mixis was reduced to 1%. A cocktail of protease inhibitors added to conditioned medium significantly reduced degradation of the mixis signal by natural proteases. Conditioned medium subjected to ultrafiltration retained mixis-inducing activity in the >10 kDa fraction, but the <10 kDa fraction had no significant activity. The putative mixis signal bound to an anion exchange column, eluting off at 0.72 M NaCl. These fractions were further separated on a C3 reversed phase HPLC column and mixis-inducing activity was associated with a 39 kDa protein. Seventeen amino acids from the N-terminus have strong similarity to a steroidogenesis-inducing protein isolated from human ovarian follicular fluid. The 39 kDa protein is an excellent candidate for the rotifer mixis induction signal.

Communicated by J.P. Grassle, New Brunswick

T. W. Snell (✉) · J. Kubanek · W. Carter · A. B. Payne
J. Kim · M. K. Hicks
School of Biology, Georgia Institute of Technology,
Atlanta 30332-0230, USA
E-mail: terry.snell@biology.gatech.edu

C.-P. Stelzer
Institute of Evolution and Biodiversity, University of Muenster,
Huefferstr. 1, 48149 Muenster, Germany

Introduction

Monogonont rotifers are cyclical parthenogens capable of both asexual and sexual reproduction (Wallace and Snell 2001). The maintenance of sex in such life cycles where it could readily be lost is a longstanding problem in evolutionary biology. Rotifers are a powerful experimental system in which to investigate this problem and the dynamic interaction between asexual and sexual reproduction, evolutionary forces, and the environment. Environmental signals such as crowding provide the stimulus to switch from asexual to sexual (mixis) reproduction in many rotifer species (Gilbert 1963, 1974, 1977, 1993, 2002, 2003a, 2004; Hino and Hirano 1976,1977; Pourriot and Snell 1983; Snell and Boyer 1988; Carmona et al 1993; Stelzer and Snell 2003; Schröder and Gilbert 2004). Crowding also acts as a stimulus for sexual reproduction in other zooplankters like *Daphnia* spp. (Stross and Hill 1965; Hobaek and Larsson 1990; Kleiven et al. 1992). Despite their ecological and evolutionary significance, neither the signal molecules nor their receptors have been characterized in any cyclical parthenogen. Clearly these molecules play a key role in understanding the evolutionary dynamics of sex, the timing of sex in life cycles, and in determining which populations participate in sexual recombination.

Natural monogonont rotifer populations typically undergo multiple generations of asexual reproduction punctuated with episodic bouts of male production and sexual recombination (Gilbert 2003b). The product of sexual reproduction is a thick-shelled resting egg (encysted embryo) that has an extended period of dormancy before hatching (Gilbert 1974, 1983). Monogonont rotifer populations in temperate climates are re-established each year by hatching from a resting egg bank in the sediments (Pourriot and Snell 1983; Gomez et al. 1995; Serra et al. 1998; Gomez et al. 2000; Ortells et al.

2000; Gomez et al. 2002b; Derry et al. 2003). After a phase of clonal propagation in the plankton, sex in brachionid rotifers is triggered by an accumulating chemical signal produced by the rotifers themselves (Gilbert 1963; Hino and Hirano 1976; Snell and Boyer 1988; Carmona et al. 1993; Stelzer and Snell 2003). Sexual females produce haploid eggs through meiosis which develop either into haploid males or, if fertilized, into resting eggs. Resting eggs can survive adverse conditions and remain viable in the sediment for decades (Marcus et al. 1994; Kotani et al. 2001; Garcia-Roger et al. 2005) and are likely a major mode of geographic dispersal (Gomez et al. 2002; Ortells et al. 2003).

Because of its importance in the rotifer life cycle, mixis has been a focus of research for more than 50 years (Gilbert 1977, 2003a). The environmental factors inducing and modifying mixis have been described (Gilbert 1977, 2003b), mictic thresholds estimated (Snell and Boyer 1988; Gilbert 2003a), the chemical nature of the signal demonstrated (Stelzer and Snell 2003), and the consequences for population dynamics determined (Serra et al. 2004). Population dynamics experiments have produced insight into the phenomenon of mixis, but advances will depend on elucidation of the molecular basis for mixis induction and the rotifer sensory systems that respond (Snell 1998). Identification of the mixis signal and its receptor(s) is especially important to fully understand the ecological and evolutionary dynamics of sex in the rotifer life cycle. Knowledge of the chemical nature of the mixis signal will provide insight into the species-specificity of the mictic response (Gilbert 2003a), the rapid loss of sex in chemostat cultures (Boraas 1983; Fussmann et al. 2003), the prevalence of both sympatric and allopatric cryptic species (Gomez et al. 2002; Suatoni et al. 2005), and the optimization of reproductive allocation in mixis (Serra et al 2004).

We used a variety of techniques to describe the chemical characteristics of the mixis signal including molecular size exclusion, selective enzymatic degradation, protease protection assays, selective binding to ion exchange media, and the N-terminal amino acid sequence. High performance liquid chromatography was used to separate active molecules, the mixis-inducing activity of fractions were evaluated using a mixis bioassay, and their protein composition was characterized using polyacrylamide gel electrophoresis. Comparing the presence and absence of proteins in active and inactive fractions allowed us to identify a few candidates for the mixis-inducing protein (MIP).

Materials and methods

Rotifer strain

The rotifers used in these experiments are part of the *Brachionus plicatilis* (Muller) species complex described by Gomez et al. (2002). The experimental population, RUS, was originally collected from the Azov Sea, Russia and is part of the Manjavacas clade as defined by phylogenetic analysis of COI and ITS genes (Gomez et al. 2002; Suatoni et al. 2005). This population has been maintained in the lab since 1983 as resting eggs. All experiments were performed with rotifers hatched from a single batch of resting eggs produced 14 September 1998 and stored at –20°C.

Rotifer culture

Rotifers were hatched from resting eggs in 15 ppt artificial seawater (ASW, Instant Ocean) at 25°C under constant fluorescent illumination of 2,000 lux. Hatchlings were fed the green alga *Tetraselmis suecica* cultured in F/2 medium (Guillard 1983) at 25°C and ASW in 5 l bags under constant fluorescent illumination.

Mixis bioassay

Resting egg hatchlings were collected for experiments under a stereomicroscope at 10X magnification using a narrow bore glass micropipet, and single rotifers 0–4 h old were transferred into 60×15 mm petri dishes with 15 ml of ASW containing 2×10^5 *T. suecica* cells ml^{-1}. All hatchlings are amictic females and reproduce parthenogenetically. Thirty-five replicate females were incubated at 23°C in darkness for 48 h on day one of the 7-day experiment. On the third day, 12 of their F1 offspring (1–15 h old) per treatment were transferred individually to new petri dishes with 15 ml ASW and algae. One group of offspring receiving no treatment was designated as the negative control. Other groups were exposed to two equal doses of conditioned medium or fractions thereof on the afternoon of the third day and on the morning of day four. Each F1 mother produced 4–8 offspring. By the afternoon of the fifth day the reproductive physiology of the F2 offspring was developmentally determined, and they were transferred individually and sequentially into wells of a 48-well plate containing 750 µl of ASW plus *T.suecica*. After the F2 offspring reproduced for two days, they were scored as mictic or amictic on day seven. Mictic females produced only male offspring, whereas amictic females produced only female offspring. This distribution of F2s in the 48-well plate allowed us to observe whether mictic offspring tended to cluster in families of the F1 mothers. We did not detect clusters of mictic daughters, rather they seemed to be randomly distributed over the plate. This suggests that if there is non-independence in mixis among F2 offspring because some were derived from the same mother, it is weak. The F2 offspring were therefore combined and analyzed as a group.

A positive control was performed by placing single F1 females into 1 ml of ASW plus 2×10^5 *T. suecica* cells in wells of a 24-well plate on day three of the bioassay. These females auto-conditioned this small volume so that

their F2 offspring were exposed to mixis-inducing signals. The F2 offspring were isolated in wells of a 48-well plate on day five and typed as mictic or amictic as described above. For each treatment, 48–96 F2 offspring were reproductively typed and the percent mixis was calculated as the percent of mictic daughters.

In the mixis bioassays the null hypothesis tested was that the frequency of mixis was independent of the treatments, which were usually some fraction of the conditioned medium. A general approach to the analysis of frequencies is a contingency table (Sokal and Rohlf 1995, p. 726). We used a Model II contingency table with one margin fixed (Sokal and Rohlf 1995, p 729) to test whether each treatment was significantly different from its control. With this design, a G test of significance is recommended. We used an alpha of 0.05 as the significance criterion, but making multiple simultaneous comparisons with 2×2 contingency tables inflates the group-wide Type I error rate (Rice 1989). As recommended by Rice, we performed a sequential Bonferroni test and significance is indicated only where the G test P value was confirmed significant by the sequential Bonferroni test.

A variation of the mixis bioassay was used to determine what development stages are responsive to the mixis signal. The bioassay protocol was identical to that described above for day one. At different times, the F1 females and/or their offspring were transferred into specially constructed Petri dishes with an outer chamber consisting of a 100×15 mm Petri dish and a smaller inner chamber of a 60×15 mm Petri dish glued into the larger dish. The inner dish had windows cut into its sides representing about 1/3 of its surface area. These windows were covered with 68-μm mesh plankton netting which allowed water exchange between the inner and outer chambers, but prevented rotifers in the inner chamber from mixing with those in the outer chamber. To facilitate diffusion, the dishes were placed on a rotary shaker moving at 12 rpm. The day before the dishes were to be used, 40 females from a log-phase culture were placed in the inner chamber in 40 ml of ASW containing 7×10^5 *T. suecica* cells ml^{-1}. Rotifers in the inner chamber conditioned the seawater producing a mixis signal equivalent to a rotifer density of about 1 rotifer ml^{-1}. Three F1 females were transferred into the outer chamber where they shared seawater with the inner chamber, but remained isolated from the rotifers inside. The F1 females and/or their offspring were transferred into the outer chamber at various times representing three stages of embryonic development of their F2 offspring. The first exposure treatment was when the single celled oocyte was developing within the mother before egg extrusion. In this treatment, F1 females were exposed from their birth on day two until day four when they began extruding eggs. The second exposure was post-extrusion when the egg was first extruded from the mother, but remained attached to her body. In this treatment, F1 females were exposed from the evening of day 4 to the morning of day 5 for a total of 15–20 h. Soon after egg extrusion, cleavage begins followed by organogenesis (Gilbert 1983). The third exposure was post-hatching when the neonate rotifer swims away from its mother and lives independently. Exposure to the mixis signal post-hatching lasted on average 24 h, from day 5 to day 6. Mixis signal exposure was controlled by transferring F1 females into or out of the outer chamber before egg extrusion, during post-extrusion, or post-hatch phases of development of their offspring. When rotifers were not being exposed, they were kept at a density of 1 female 15 ml^{-1}, which is a non-mixis-inducing environment. When the F2 offspring hatched after exposure was completed, they were transferred individually into wells of a 48-well plate containing 750 μl of ASW plus *T. suecica*. After two days, 48–96 F2 offspring were scored as mictic or amictic and the percent mixis was calculated. A Fisher's exact test was calculated to estimate the probability that treatments differed significantly from negative and positive controls. To determine the exact length of each phase of development, several rotifers were cultured individually from birth and checked hourly to determine the time of first egg extrusion and first hatching.

Conditioned medium

Conditioned medium was prepared by filtering large numbers of rotifers from mass cultures. We raised rotifers in ASW in 240-l cylinders at 22°C with constant fluorescent illumination on a diet of *T. suecica*. When rotifer densities reached about 20 ml^{-1}, the culture was filtered over 68-μm mesh Nitex screen which retained the rotifers. This biomass typically contained $10–20\times10^6$ rotifers and was re-suspended in 1.2 l of fresh ASW and lightly aerated for 4-h. After this period of conditioning, rotifers were removed by filtration over 44-μm and then 35-μm Nitex. The conditioned medium was frozen at −20°C for a few days until experimental separations were initiated. Conditioned medium was tested in the mixis assay at its natural concentration in lab cultures—that is, each rotifer was placed in 15 ml of unconcentrated conditioned medium, whereas in negative controls each rotifer was placed in 15 ml of ASW.

Ultrafiltration of conditioned medium

The conditioned medium was thawed and placed at 4°C in a stirred ultrafiltration cell (Amicon) with a 10,000 molecular weight cut-off membrane (Millipore). The 1.2 l of medium was filtered over several hours until the volume was reduced to about 80 ml (16-fold concentration). The retentate (> 10 kDa) was centrifuged at 3,700 g for 5 min to remove particulates, and both retentate and eluate were frozen at −20°C in 40-ml aliquots for later ion exchange chromatography. Prior to chromatography, the sample was ultrafiltered through a

100,000 molecular weight cut-off membrane and the retentate (>100 kDa fraction) discarded.

Protease digestion and protease inhibition

Conditioned medium was exposed to 1.3 U of proteinase K (Sigma P−2308) for 60 min at 37°C; controls lacked the enzyme. Proteinase K at a concentration of 1 U ml^{-1} was added to auto-conditioned medium with a density of 1 female ml^{-1} (positive control) and incubated at 23°C for 48 h. A cocktail of protease inhibitors (Roche, Mini Complete, #1 836 153, one tablet 7 ml^{-1}) was added to the conditioned medium. According to the manufacturer, this cocktail inhibits a broad spectrum of serine-, cysteine- and metallo-proteases.

Ion exchange chromatography

Twenty five milliliters of the concentrated conditioned medium was applied to 3 ml of super Q anion exchange resin (Amersham) packed in a 2-cm diameter glass column. Proteins were eluted in a pH 8.0, 20 mM Tris buffer with a linear gradient from 0.2 to 1 M NaCl over 100 min at a flow rate of 0.7 ml min^{-1}. Proteins were detected by UV absorption at 280 nm. Activity typically eluted off in the 55–110 min fraction (40-ml volume) and one Roche Mini Complete protease inhibitor tablet was added prior to storage at −20°C. The sample was defrosted and concentrated to 2 ml by ultrafiltration (10 kDa) just before testing for mixis-inducing activity. Mixis-inducing activity was assayed by injecting 40 µl of each fraction directly into a petri dish containing 15 ml ASW and one female rotifer. A second dose of 40 µl of the appropriate fraction was added to each petri dish on the following day. Relative to the original conditioned medium, each ion exchange fraction was tested at approximately 50% of the original concentration (i.e., the steps leading up to this point constituted a concentration factor of 200-fold, whereas each ion exchange fraction was applied in the bioassay with a dilution factor of 375-fold, resulting in an overall dilution factor of 1.9-fold).

High performance liquid chromatography

Two 1-ml samples of the concentrated ion exchange fraction eluting between 55–110 min were consecutively injected into an Agilent SB-C3 reversed-phase 4.6×250 mm column (Agilent 880975-909) on a Waters 1525 gradient HPLC system fitted with a Waters 2996 photodiode array detector or Waters 2487 tunable dual wavelength UV detector, operated with Waters Breeze software. Proteins were eluted with a linear gradient of water and acetonitrile over 25 min, at a flow rate of 1 ml min^{-1}. Proteins were detected by UV absorption at 280 nm and peaks were collected in various volumes. HPLC fractions from the two 1-ml samples were combined based upon common retention times. Samples were placed in a −80°C freezer for 1 h and then freeze-dried overnight. Samples were redissolved in 100 µl of deionized water, half of which was diluted to 500 µl with deionized water and used for the mixis bioassay as described above. Thus, each HPLC fraction was tested at approximately the original concentration in conditioned medium (i.e., the steps leading up to this point constituted a concentration factor of 400-fold, whereas each HPLC fraction was applied in the bioassay with a dilution factor of 375-fold). The remainder of the sample was used for PAGE electrophoresis.

Polyacrylamide gel electrophoresis

Soluble proteins were separated and visualized by SDS-polyacrylamide gel electrophoresis performed according to Snell et al. (1995). Six microliters of electrophoresis sample solution were added to 18 µl of each HPLC fraction and heated at 100°C for 5 min. Proteins were separated on 12% acrylamide gels and visualized with Sypro stain (Molecular Probes) according to the manufacturer's protocol. The detection limit of Sypro stain is approximately 25 ng protein.

Results

Mixis induction by auto-conditioning

Single females isolated in small volumes produce enough chemical signal to trigger mixis in their F1 offspring. We investigated the culture volume necessary to prevent accumulation of the mixis signal to threshold levels inducing mixis (Fig. 1). A single female was maintained in 1 ml ASW with algae but without transfer to fresh medium for 2 days. Her F1 offspring were exposed to this conditioned medium through their embryonic development and on average 17% of daughters became

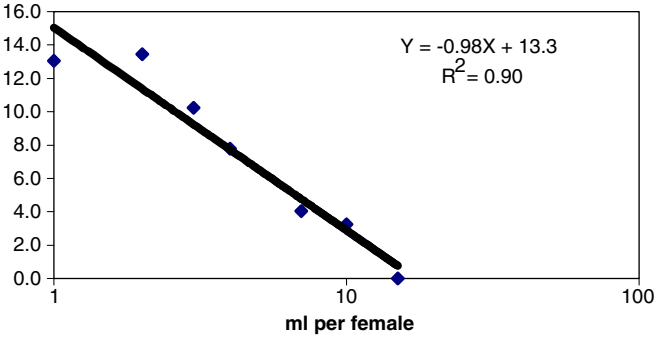

Fig. 1 *Brachionus plicatilis*. Threshold density triggering mixis by auto-conditioning of medium. *X*-axis refers to volume in which a single female was cultured for 48 h and *Y*-axis the percent of offspring that were mictic

mictic. In contrast, if a single female was cultured in 10 ml, only 3% of her offspring became mictic. Culturing a female in 15 ml diluted the mixis signal to below the threshold concentration triggering mixis. A linear regression of percent mixis on log culture volume yielded an R^2 of 0.9 and a mixis threshold of 1 female 14 ml^{-1} (71 l^{-1}) (regression ANOVA: $F = 26.5$, $P = 0.002$). We cultured a single female in 15 ml as the negative control for all subsequent mixis bioassays. In ten replicate experiments over two months, negative controls averaged 1.9% mixis with 95% confidence limits of 2.9–0.8%. Positive controls (1 female ml^{-1}) averaged 16.8% mixis with 95% confidence limits of 21.4–12.3%.

We also tested the mixis-inducing ability of the conditioned medium derived from the mass cultures. When a single F1 female was cultured in 18 ml of the conditioned medium, 22% of her daughters became mictic ($n = 91$). If this conditioned medium was diluted to 5% with ASW, 11.3% of daughters were mictic ($n = 53$). This clearly demonstrates that conditioned medium induces mixis, even when rotifers are cultured at very low densities.

Protein nature of the mixis signal

If the mixis signal were a protein, we expected it to be degraded by protease enzymes (proteinase K) and protected from degradation by natural proteases by protease inhibitors (Fig. 2). When conditioned medium (CM) was exposed to proteinase K, its mixis-inducing ability was reduced 70% (CM vs. CM + protK, G test = 3.99, $P = 0.046$). Likewise, when proteinase K was added to the auto-conditioned positive control (1 female ml^{-1}), where typically 17% of daughters became mictic, mixis was reduced to 1% (G test = 16.3, $P < 0.0001$). In comparison, when a cocktail of protease inhibitors was added to the conditioned medium, degradation of the mixis signal by natural proteases was reduced (Fig. 2). The addition of proteinase K did not reduce the reproductive rate of the maternal females. Reduced mixis in this treatment therefore must be regarded as a direct effect on the mixis protein rather than an indirect effect through lowered reproductive rates. The data in Fig. 2 were collected in four separate experiments over one month. Each had its own negative and positive controls which were pooled after a G test revealed no significant differences among them.

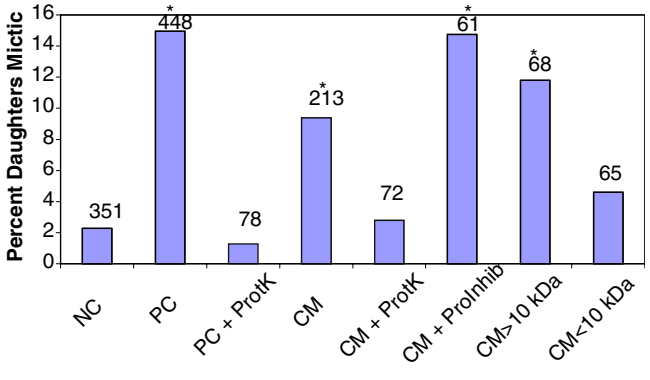

Fig. 2 *Brachionus plicatilis*. Effect of proteinase K and protease inhibitors on mixis signal in conditioned medium. *NC* negative control; *PC* auto-conditioned positive control; *PC + protK* proteinase K added to the positive control; *CM* untreated conditioned medium; *CM + protK* conditioned medium treated with proteinase K; *CM + proinhib* treated with protease inhibitors. CM > 10 kDa is the fraction of conditioned medium retained on a 10 kDa molecular weight cutoff membrane and CM < 10 kDa is fraction that passed through. Negative and positive controls were single females cultured in 15 and 1 ml of untreated ASW, respectively. *Numbers on top of bars* are the number of females scored for mixis (n). Treatments with an asterisk (*) were significantly greater than the negative control by G test

Size of the mixis signal molecule

The size of the mixis signal molecule was estimated by fractionating with membranes with pore sizes of various molecular weights (Fig. 2). The mixis-inducing ability of the > 10 kDa fraction was not significantly different from the auto-conditioned positive control ($G = 0.508$, $P = 0.476$), whereas that of the < 10 kDa fraction was similar to the negative control ($G = 0.996$, $P = 0.318$), indicating that the mixis signal is > 10 kDa. We later incorporated a 100 kDa filtration step in the purification scheme and the mixis signal was in the flow through (data not shown).

Ion exchange fractions containing the mixis signal

Retention of the mixis signal on an anion column is illustrated in Fig. 3. As the salt concentration reached about 0.72 M after 70 min, mixis signal proteins began to elute off. The 72–87 min and 87–115 min fractions had tenfold more mixis-inducing activity than the neg-

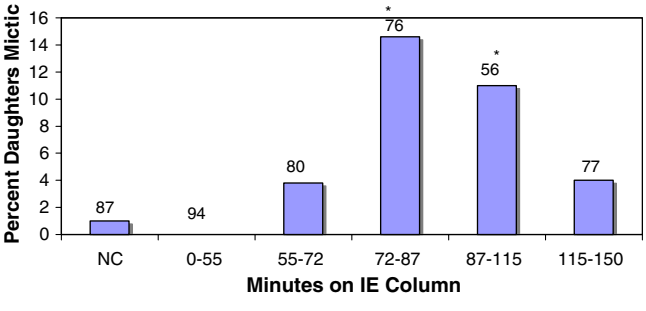

Fig. 3 *Brachionus plicatilis*. Binding of mixis signal to an ion exchange column. Minutes on IE column refers to time each fraction was retained on ion exchange column before eluting off in a linear salt gradient of 0.2–1 M. Numbers over bars are numbers of females scored for mixis (n). Percent daughters mictic is the percentage of mictic offspring from females exposed to various fractions in a mixis bioassay. Fractions designated with an asterisk (*) were significantly greater than the negative control by G test

ative control ($G = 12.1$ and 6.91, $P = 0.0005$ and 0.0086, respectively).

HPLC fractions containing the mixis signal

The HPLC chromatogram revealed ten major peaks eluting after 10–30 min on the column (Fig. 4a). All of these were tested for mixis-inducing activity, which was consistently associated with the fraction eluting at 17–19 min (Fig. 4b). The only fractions with significantly higher mixis than the negative control were the positive control ($G = 5.19$, $P = 0.016$) and the 17–19 min fraction ($G = 5.84$, $P = 0.016$). The 17–19 min fraction induced 90% as much mixis as the auto-induced positive control. Two major peaks are present in this fraction, one eluting at 17.4 min and a second eluting at 18.6 min. These were tested separately in several replicate experiments. In some experiments significant mixis-inducing activity was found in the 17.4 min peak and in others it was associated with the 18.6 min peak (data not shown). In subsequent bioassays we therefore tested the combined 17–19 min fraction.

Polyacrylamide gel electrophoresis of HPLC fractions

The HPLC fractions differed markedly in their protein composition (Fig. 5). The 11–14 min fraction contained a single dominant band with an apparent molecular mass of about 100 kDa. The 14–17 min fraction contained five visible bands of 100, 77, 74, 45, and 39 kDa. The 17–18 min fraction had only 45 and 39 kDa bands and the 18–19 min fraction had only 39 kDa. Fractions 19–21, 23–25, and 28–30 contained no visibly detectable proteins. The 39 kDa protein(s) eluted in three fractions (14–17, 17–18, 18–19), but not all fractions with a 39 kDa band possessed significant mixis-inducing activity (the 14–17 min fraction lacked activity). However, we never observed significant mixis-inducing activity without a 39 kDa band present (fractions 17–18, 18–19).

N-terminal amino acid sequence of the 39 kDa protein

The 39 kDa protein band was Western blotted from a PAGE gel and brought to the Emory University microsequencing facility for N-terminal sequencing. Edman N-terminal degradation sequencing was performed on the 39 kD protein, yielding a signal at the 0.7 pmol level. The N-terminal sequence was determined to be dVNGGGATLPQpLYQTA. Residues in lowercase were tentatively assigned; in cycle #12, proline yielded a weak signal indicating that other unstable residues (e.g. Trp or Cys) or a modified residue could be present in this position. A BLAST analysis performed on this sequence revealed 100% identity ($P = 0.00084$) to the N-terminal fragment of the steroidogenesis-inducing protein (SIP) (Genbank accession number P83897) isolated from human ovarian follicular fluid.

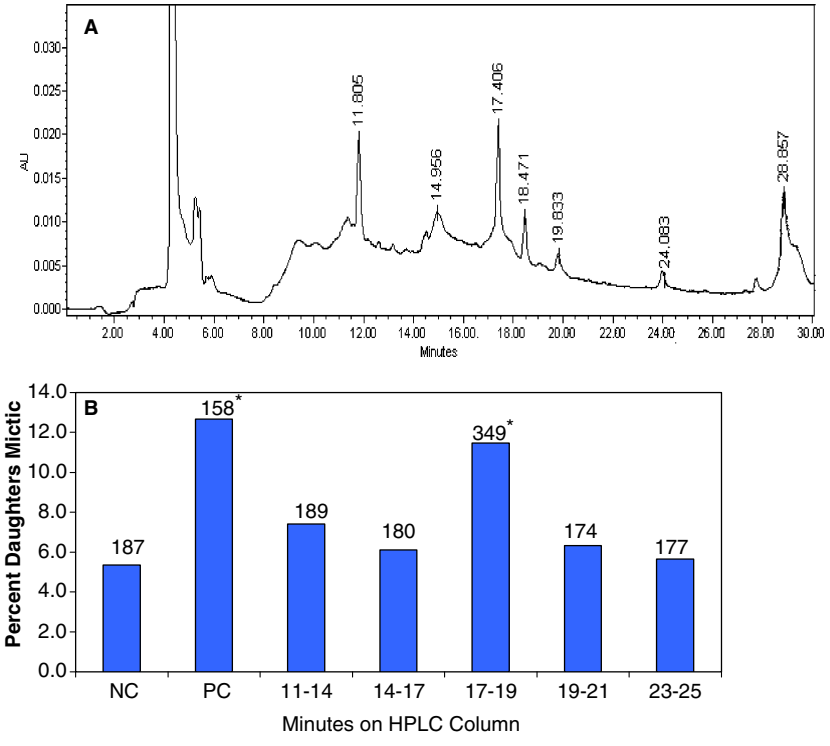

Fig. 4 *Brachionus plicatilis*. **a** HPLC chromatogram. Column retention time in minutes is on *X*-axis and absorbance units (*AU*) at 280 nm are on *Y*-axis. Numbers at peak maxima are retention times in minutes. **b** Mixis activity in HPLC fractions. Minutes on HPLC column are retention times, *numbers on bars* are numbers of females scored for mixis (*n*). Negative (*NC*) and positive (*PC*) controls were single females cultured in 15 and 1 ml of untreated ASW, respectively. Treatments marked with an *asterisk* were significantly greater than negative control by *G* test

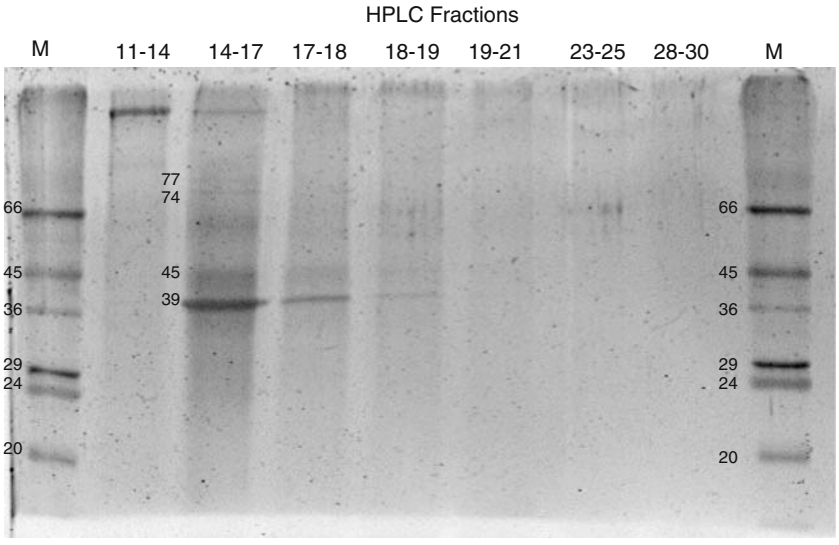

Fig. 5 *Brachionus plicatilis*. SDS-polyacrylamide gel of HPLC fractions. Names above each lane represent minutes each fraction was retained on HPLC column. *M* molecular weight marker lane. *Numbers to the left of the protein bands* are molecular weight estimates in kilodalton. Proteins are visualized with Sypro stain

Developmental stage responsive to the mictic signal

Embryonic development of *B. plicatilis* was divided into before egg extrusion, post-egg extrusion, and post-hatch phases. At 23°C, the before egg extrusion phase lasted 18.3 ± 0.60 h and post-extrusion for 14.8 ± 0.62 h. The post-hatch phase represents the first 24 h after hatching. Exposure to the conditioned medium only in the before egg extrusion phase induced more than tenfold higher mixis than the negative control (Fig. 6, $G = 10.5$, $P = 0.0012$). In contrast, exposure only during the post-extrusion or post-hatch phases did not induce significantly more mixis than the negative control ($G = 1.66$ and 1.58, $P = 0.198$ and 0.209, respectively). Exposure to the conditioned medium during both the before egg extrusion and post-extrusion phases induced significantly more mixis than observed in the negative control ($G = 6.85$, $P = 0.010$), but no more mixis than before egg extrusion only exposure. Likewise, exposure to conditioned medium during before egg extrusion and post-hatch phases induced significantly more mixis than the negative control ($G = 12.4$, $P = 0.0004$), but no more mixis than the before egg extrusion only exposure. Exposure to conditioned medium during post-extrusion and post-hatch phases did not induce significantly more mixis over that of the negative control ($G = 0.047$, $P = 0.828$).

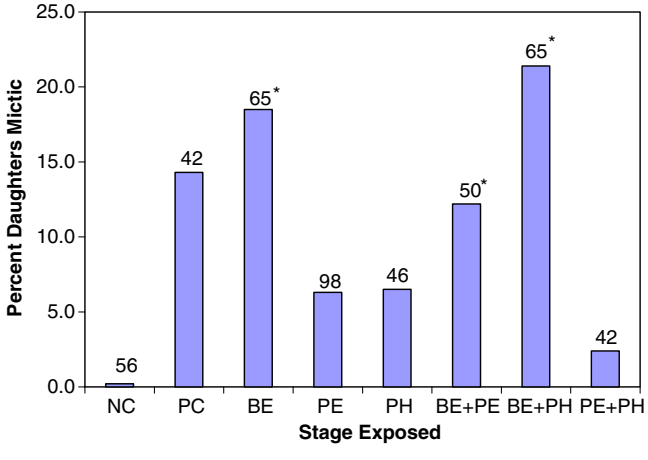

Fig. 6 *Brachionus plicatilis*. Effect of exposure to the mixis signal at various stages of embryonic development. *X*-axis is stage exposed: *NC* negative control; *PC* positive control; *BE* before egg extrusion; *PE* post-egg extrusion; *PH* post-egg hatch. Negative and positive controls were single females cultured in 15 and 1 ml of untreated ASW, respectively. *Numbers on bars* are number of females scored for mixis (*n*). Treatments labeled with an *asterisk* (*) were significantly greater than the negative control by *G* test

Discussion and conclusions

Our study shows that the mixis-inducing signal is a protein (MIP) smaller than 100 kDa and larger than 10 kDa. It is degraded by proteases and protected from degradation by natural proteases by adding protease inhibitors. The ability of proteinase K to reduce mixis in the auto-conditioned positive control is especially revealing. In this treatment, single females in 1 ml ASW produced enough mixis signal that typically 17% of their offspring became mictic. Adding proteinase K to this medium selectively degraded enough protein to abolish the mictic signal, even though the signal was presumably constantly produced by the maternal female and her progeny. Developing oocytes were not exposed to the external medium until their extrusion as eggs, yet they were the most sensitive to the mixis signal. This suggests that the mixis signal does not interact directly with the oocyte before extrusion, but likely binds to a receptor on the mother, eliciting an internal signal that is transmitted to the oocyte. These observations strongly suggest that proteins in conditioned medium are a necessary and sufficient mixis induction signal.

The binding of the MIP to an anion exchange column suggests that the molecule possesses a net negative charge at pH 8.0 (the pH at which the ion exchange chromatography was conducted), which is reasonable but not particularly diagnostic for proteins. The adsorption of the MIP to a C3 reversed-phase HPLC column, eluting off at 60–70% acetonitrile, also is consistent with a protein with some hydrophobic domains. The repeated association of a 39 kDa protein with mixis-inducing activity focuses attention on this molecule as a prime candidate for the signal. However, the absence of significant mixis-inducing activity in some samples with a 39 kDa protein requires explanation. We hypothesize that the 39 kDa protein is transformed from active to inactive forms without a change in mass detectable by PAGE electrophoresis. Transformation to an inactive structure could occur, for example, by breaking of disulfide bonds, by selective oxidation/reduction, or by cleavage of one or a few amino acids. If this hypothesis is true, it explains why the active HPLC fraction elutes as two peaks. These peaks would thus represent two closely related, MIPs of similar mass, but with slightly different hydrophobicities.

The sequence of 17 amino acids from the N-terminus of the 39 kDa protein was identical to the human steroidogenesis-inducing protein (SIP) (Kahn et al. 2005). The metabolic role of this protein is not fully understood, but one of its functions is to regulate reproductive steroid hormone synthesis in human ovaries. If rotifers are using a similar steroidogenesis-inducing protein to regulate mixis, this has far reaching implications for the phylogenetic conservation of reproductive hormones as well as for the hormonal basis of sex induction in rotifers.

In the species of rotifers thus far investigated, the mictic or amictic fate of a female is determined as the egg develops inside the maternal female, before egg extrusion (Shull 1912; Buchner 1941; Ruttner-Kolisko 1964). To explore reproductive determination in *B. plicatilis*, we separated rotifer development into three distinct phases. The before egg extrusion phase is initiated by the birth of the maternal female. Rotifers are eutelic, lacking cell division as adults (Gilbert 1988), so females are born with all of the oocytes that they will develop as adults. As a maternal female matures into a reproductive adult, her oocytes develop sequentially, with yolk supplied by the vitellarium (Gilbert 1983). During this phase, the embryo is sequestered in the pseudocoelom of the maternal female without exposure to the external medium. Once an egg receives sufficient yolk, it is extruded and carried by the female until hatching. At 23°C, the first egg of *B. plicatilis* is extruded when the maternal female is on average 18.3 h old. Amictic eggs are large relative to maternal females, on the order of 50% of female length (Snell and Hoff 1987). Extrusion is the embryo's first exposure to the external environment and, after being carried by the maternal female for on average 14.8 h, eggs hatch and a free-swimming neonate emerges. Our data suggest that *B. plicatilis* females are most responsive to the mixis signal as oocytes inside their mothers and that exposure to mixis signals later in development has little effect.

A protein signal regulating mixis has the advantage of high signal specificity, narrow response time window, and low detection limits. Signal protein-receptor binding can provide high specificity that can be recognized in a sea of noise. It is critical that a rotifer population be unresponsive to all but conspecific mixis signals so that they can successfully produce resting eggs. For example, from the first resting egg that hatches in the spring, the population can grow, producing an increasing concentration of mixis signal until the mixis threshold is reached. This population would be responding to its own mixis signal and appropriately initiating mixis at high population density. In contrast, a later hatching resting egg from a closely related species hatches into an environment where mixis signal from another species already exceeds threshold. Responding to this heterospecific signal would trigger mixis prematurely, resulting in mictic female production at inappropriately low population density (Gilbert 2003a). This would lead to much lower resting egg production than if mixis were initiated at higher population densities (Snell 1987; Serra and King 1999). Response to a heterospecific mixis signal would therefore likely reduce the long term fitness of a population (Serra et al. 2004). Employing a protein as the mictic signal could provide the specificity necessary to restrict species responsiveness to conspecific mictic cues, even in the presence of closely related species.

A protein mixis signal allows for a narrow response time window so that signal strength is highly correlated with population density. The sensitivity of the mixis signal protein to degradation by natural proteases requires constant production to maintain signal concentration above threshold and allows for its rapid elimination when population density falls below threshold. A protein mixis signal also permits a very low mixis threshold, because binding of a protein to a receptor allows the signal to be amplified via signal transduction pathways, which are well characterized for invertebrates (Krieger and Breer 1999). The threshold density for mixis appears to be very low, since a single *B. plicatilis* female in less than 15 ml for 48 h produces enough signal to elicit mixis in her offspring. This is equivalent to a predicted mixis threshold of 71 rotifers l^{-1} in natural populations and suggests that a rotifer with a volume of about 0.008 cm^3 can quickly produce enough signal to induce mixis in a volume 1910 times its size. Carmona et al. (1995) reported a mixis threshold of 6.6 l^{-1} for *B. plicatilis* and 23 l^{-1} for *B. rotundiformis*, which co-occur in Torreblanca, Spain. Gilbert (2003a) reported a mictic threshold of 67–167 for the freshwater congener *B. calyciflorus*. Serra et al. (2005) showed that rotifer populations with a mixis ratio of 14% and a mixis threshold of 70 l^{-1} were evolutionarily stable. They could invade other populations with different mixis patterns, but were resistant to invasion by genotypes with different mixis patterns.

Identifying the mixis signal as a protein explains several observations about sexual reproduction in rotifers. Gilbert (2003a) documented the species-specificity of the mixis signal by observing that *B. calyciflorus* populations from Florida and Georgia initiated mixis when crowded with members of the same population, but were unresponsive to crowding by a *B. calyciflorus* population from Australia. He concluded that the mixis signal must be differentiated between these populations. In contrast, Stelzer and Snell (2005) have shown that the mixis induction signal may be little differentiated in the *B. plicatilis* species complex. In a group of five closely related species, conditioned medium from all but one species was capable of inducing mixis in heterospecifics. Clearly a broader investigation of differentiation in the mixis signal is required before there is a general understanding of the species-specificity of mixis induction. The chemical characterization of the MIP presented here will facilitate such studies as it has for quorum sensing by bacteria which is achieved through the highly specific binding of signal molecules to receptors as illustrated by the auto-inducing polypeptides of gram positives (Taga and Bassler 2003).

Some rotifer species experience several less responsive generations following hatching from resting eggs before induction of mixis (Gilbert 2002; Schröder and Gilbert 2004). The phenomenon was first described in *B. calyciflorus* where females hatching from resting eggs became fully responsive to the mixis signal only after 5–12 asexual generations (Gilbert 2003a). Other rotifer species like *B. angularis, B. variabilis, Epiphanes senta*, and *Rhinoglena frontalis* also exhibit delayed mixis, but there is considerable variability among populations in the amount of delay (Gilbert 2002; Schröder and Gilbert 2004). It is not known whether *B. plicatilis* also has delayed mixis, but our observation that there is 17% mixis in the F2 generation after resting egg hatching suggests that, if present, it is weak. The pattern of mixis in the *B. plicatilis* Russian strain is similar to that observed in a Georgia population of *B. calyciflorus* (40% mixis in the F2) and *B. angularis* (12% mixis in the F2) (Schröder and Gilbert 2004). Moreover, as coastal marine and salt lake species, many *B. plicatilis* populations inhabit permanent water bodies, whereas rotifer populations from temporary habitats are the most likely to have delayed mixis (Gilbert 2002; Schröder and Gilbert 2004). Delayed mixis seems to be an adaptive response to prematurely initiating mixis in response to mictic signals from conspecifics (Serra et al. 2005). If the mixis signal is a protein, a possible mechanism for reversibly regulating responsiveness to a mixis signal is provided. Mixis signal production could be reduced in females recently hatched from resting eggs by suppressing the expression of a single gene coding for the MIP.

A third phenomenon that could be explained if the mixis signal is a protein is the rapid loss of sex in chemostats. Experimental selection can reproducibly produce obligate parthenogens in laboratory chemostats in a few months (20–30 generations) after collection from natural populations. Boraas (1983) found that newly established cultures of *B. calyciflorus* collected from the field produced 40% mictic females when induced, but that after 2–3 months in a chemostat, mixis was eliminated. He argued that the rapid loss of mixis was permanent, due to selection against sexual reproduction. More recently, Fussmann et al. (2003) speculated that selection against mictic reproduction occurs annually (through the growing season) in natural environments, but is balanced by elimination of obligate asexual individuals at the end of the year. These authors argued that the dynamics of natural rotifer populations can only be understood by considering rapid evolutionary change as part of their demographics and that evolutionary and ecological time scales overlap (see also Yoshida et al. 2003). Knowing that the mixis signal is a protein helps clarify the mechanisms underlying these dynamics. Our current model of mixis loss postulates that mutations appear somewhere in the mixis signaling pathway. This pathway probably includes genes involved in MIP production, chemoreception, *G*-protein signaling, intracellular messengers, and/or transcription factors. None of these genes has been characterized in rotifers, but analogous pathways are involved in the chemosensory mating behavior of *Drosophila* spp. (Bray and Amrein 2003). Reduced production of the MIP and loss of sensitivity in the receptors are prime candidates for the mechanism underlying the rapid loss of mixis in chemostats since these steps are at the beginning of the pathway. If the mixis signal is a protein, it is clear that mutations in one or a few genes could alter mictic signal production or chemoreception.

Cryptic species complexes are common in monogonont rotifers (Serra et al. 1997; Gomez et al. 2002; Derry et al. 2003; Suatoni et al. 2005; Gilbert and Walsh 2005), and this can be partially explained if the mixis signal is a protein. Species boundaries in rotifers often are unrecognized because they are based on chemical communication (Snell 1989, 1998). Most rotifer taxonomy is still based on morphology (Segers 1998, 2002), so many reproductively isolated taxa are undoubtedly lumped into a single species (Suatoni et al. 2005). This raises the question of how morphologically very similar species coexist and maintain species integrity? It is well known that rotifer mate recognition systems contribute substantially to the reproductive barriers between species (Snell 1989). It is less appreciated, however, that differential responsiveness to mixis signals can likewise maintain barriers to interspecific hybridization by temporally separating sexual reproduction (Gilbert 2003b). Small alterations to MIPs or to receptors involved in their detection could lead to reproductive isolation among closely related species or even diverging populations. As we understand more about rotifer mixis-induction systems, it is clear how the specificity of the mixis response can allow two closely related species to co-occur, yet maintain non-overlapping periods of sexual reproduction. Temporal separation of sexual periods, along with strong mate recognition systems are

enough to allow very similar morphospecies to coexist without hybridization.

Acknowledgements We thank John Gilbert, Manuel Serra, and David Mark Welch for valuable comments that improved this paper. This work was supported by the National Science Foundation grants BE/GenEn MCB-0412674 to TWS and OCE-0134843 to JK. The Deutsche Forschungsgemeinschaft (STE 1021/1) supported CPS with a post-doctoral fellowship.

References

Boraas ME (1983) Population dynamics of food limited rotifers in two-stage chemostat culture. Limnol Oceanogr 28:546–563

Bray S, Amrein H (2003) A putative *Drosophila* pheromone receptor expressed in male-specific taste neurons is required for efficient courtship. Neuron 39:1019–1029

Buchner H (1941) Entwicklungsphyiologische Untersuchungen uber den Generationswechsel der Radertiere. 1. Einleitende Studien uber den Determinationspunkt. Wilhelm Roux Arch Entwicklung Organ 141:154–158

Carmona MJ, Serra M, Miracle MR (1993) Relationships between mixis in *Brachionus plicatilis* and preconditioning of culture medium by crowding. Hydrobiologia 255/256:145–152

Carmona MJ, Gomez A, Serra M (1995) Mictic patterns of *Brachionus plicatilis* in small ponds. Hydrobiologia 313/314:365–371

Derry AM, Hebert PDN, Prepas EE (2003) Evolution of rotifers in saline and subsaline lakes: A molecular phylogenetic approach. Limnol Oceanogr 48:675–685

Fussmann GF, Ellner SP, Hairston NG Jr. (2003) Evolution as a critical component of plankton dynamics. Proc R Soc Lond B 270:1015–1022

Garcia-Roger EM, Carmona MJ, Serra M (2005) Deterioration patterns in diapausing egg banks of *Brachionus* (Muller, 1786) rotifer species. J Exp Mar Biol Ecol 314:149–161

Gilbert JJ (1963) Mictic female production in the rotifer *Brachionus calyciflorus*. J Exp Zool 153:113–124

Gilbert JJ (1974) Dormancy in rotifers. Trans Am Microsc Soc 93:490–513

Gilbert JJ (1977) Mictic-female production in monogonont rotifers. Archiv für Hydrobiologie Beihefte 8:142–155

Gilbert JJ (1983) Rotifera. In: Adiyodi KG, Adiyodi RG (eds) Reproductive biology of invertebrates, vol I. Oogenesis, oviposition, and oosorption. Wiley, New York, pp 181–209

Gilbert JJ (1988) Rotifera. In: Adiyodi KG, Adiyodi RG (eds) Reproductive biology of invertebrates, vol IV, part A. Fertilization, development, and parental care. Oxford and IBH Publishing Company, New Delhi, pp 179–199

Gilbert JJ (1993) Rotifera. In: Adiyodi KG, Adiyodi RG (eds) Reproductive biology of invertebrates, vol VI, part A. Asexual propagation and reproductive strategies. Oxford and IBH Publishing Company, New Delhi, pp 231–263

Gilbert JJ (2002) Natural regulation of environmentally-induced sexuality in a rotifer: a multigenerational parental effect induced by fertilization. Freshw Biol 47:1633–1641

Gilbert JJ (2003a) Specificity of crowding response that induces sexuality in the rotifer *Brachionus*. Limnol Oceangr 48:1297–1303

Gilbert JJ (2003b) Environmental and natural control of sexuality in a rotifer life cycle: developmental and population biology. Evol Dev 5:19–24

Gilbert JJ (2004) Population density, sexual reproduction and diapause in monogonont rotifers: new data for *Brachionus* and a review. J Limnol 63(suppl):32–36

Gilbert JJ, Schröder T (2004) Rotifers from diapausing, fertilized eggs: Unique features and emergence. Limnol Oceanogr 49:1341–1354

Gilbert JJ, Walsh EJ (2005) *Brachionus calyciflorus* is a species complex: Mating behavior and genetic differentiation among four geographically isolated strains. Hydrobiologia 546:257–265

Gómez A, Temprano M, Serra M (1995) Ecological genetics of a cyclical parthenogen in temporary habitats. J Evol Biol 8:601–622

Gómez A, Carvalho GR, Lunt DH (2000) Phylogeography and regional endemism of a passively dispersing zooplankter: mtDNA variation of rotifer resting egg banks. Proc R Soc Lond B 267:2189–2197

Gómez A, Serra M, Carvalho GR, Lunt DH (2002) Speciation in ancient cryptic species complexes: evidence from the molecular phylogeny of *Brachionus plicatilis* (Rotifera). Evolution 56:1431–1444

Gómez A, Adcock GA, Lunt DH, Carvalho GR (2002b) The interplay between colonization history and gene flow in passively dispersing zooplankton: microsatellite analysis of rotifer resting egg banks. J Evol Biol 15:158–171

Guillard RRL (1983) Culture of phytoplankton for feeding marine invertebrates. In: Berg CJ Jr (ed) Culture of marine invertebrates. Hutchinson Ross, Stroudsburg

Hino A, Hirano R (1976) Ecological studies on the mechanism of bisexual reproduction in the rotifer *Brachionus plicatilis*—I. General aspects of bisexual reproduction inducing factors. Bull Jpn Soc Sci Fish 42:1093–1099

Hino A, Hirano R (1977) Ecological studies on the mechanism of bisexual reproduction in the rotifer Brachionus plicatilis—II. Effects of cumulative parthenogenetic generation on the frequency of bisexual reproduction. Bull Jpn Soc Sci Fish 43:1147–1155

Hobæk A, Larsson P (1990) Sex determination in *Daphnia magna*. Ecology 71:2255–2268

Kahn S, May P, Matisiak E, Reddy J, Konu O, Cheng Z, Millena A (2005) Isolation of human steroidogenesis-inducing protein from human ovarian follicular fluid (submitted)

Kleiven OT, Larsson P, Hobæk A (1992) Sexual reproduction in *Daphnia magna* requires three stimuli. Oikos 65:197–206

Kotani T, Ozaki M, Matsuoka K, Snell TW, Hagiwara A (2001) Reproductive isolation among geographically and temporally isolated marine *Brachionus* populations. Hydrobiologia 446/447:283–290

Krieger J, Breer H (1999) Olfactory reception in invertebrates. Science 286:720–723

Marcus NH, Lutz R, Burnett W, Cable P (1994) Age, viability and vertical distribution of zooplankton resting eggs from an anoxic basin: evidence of an egg bank. Limnol Oceanogr 39:154–158

Ortells R, Snell TW, Gómez A, Serra M (2000) Patterns of genetic differentiation in resting egg banks of a rotifer species complex in Spain. Arch Hydrobiol 149:529–551

Ortells R, Gómez A, Serra M (2003) Coexistence of cryptic rotifer species: ecological and genetic characterization of *Brachionus plicatilis*. Freshw Biol 48: 2194–2202

Pourriot R, Snell TW (1983) Resting eggs in rotifers. Hydrobiologia 104:213–224

Rice WR (1989) Analyzing tables of statistical tests. Evolution 43:223–225

Ruttner-Kolisko A (1964) Uber der labile Periode im Fortpflanzungszyklus der Radertiere. Int Gesam Hydrobiol 49:473–482

Schröder T, Gilbert JJ (2004) Transgenerational plasticity for sexual reproduction and diapause in the life cycle of monogonont rotifers: Intraclonal, intraspecific and interspecific variation in the response to crowding. Funct Ecol 18:458–466

Segers H (1998) An analysis of taxonomic studies on Rotifera: a case study. Hydrobiologia 387/388:9–14

Segers H (2002) The nomenclature of the Rotifera: annotated checklist of valid family-and genus-group names. J Nat Hist 36:631–640

Serra M, King CE (1999) Optimal rates of bisexual reproduction in cyclical parthenogens with density-dependent growth. J Evol Biol 12:263–271

Serra M, Galiana A, Gómez A (1997) Speciation in monogonont rotifers. Hydrobiologia 358:63–70
Serra M, Gómez A, Carmona MJ (1998) Ecological genetics of *Brachionus* sibling species. Hydrobiologia 387/388:373–384
Serra M, Snell TW, King CE (2004) The timing of sex in cyclically parthenogenetic rotifers. In: Moya A, Font E (eds) Evolution: from molecules to ecosystems. Oxford University Press, New York, pp 135–146
Serra M, Snell TW, Gilbert JJ (2005) Delayed mixis in rotifers: an adaptive response to the effects of density-dependent sex on population growth. J Plankton Res 27:37–45
Shull AF (1912) Studies in the life cycle of *Hydatina senta*. III. Internal factors influencing the proportion of male-producers. J Exp Zool 12:283–317
Snell TW (1987) Sex, population dynamics and resting egg production in rotifers. Hydrobiologia 144:105–111
Snell TW (1989) Systematics, reproductive isolation and species boundaries in monogonont rotifers. Hydrobiologia 186/187:299–310
Snell TW (1998) Chemical ecology of rotifers. Hydrobiologia 387/388:267–276
Snell TW, Hoff FH (1987) Fertilization and male fertility in the rotifer *Brachionus plicatilis*. Hydrobiologia 147:329–334
Sokal RR, Rohlf FJ (1995) Biometry. W.H. Freeman, New York
Stelzer CP, Snell TW (2003) Induction of sexual reproduction in *Brachionus plicatilis* (Monogononta, Rotifera) by a density-dependent chemical cue. Limnol Oceanogr 48:939–943
Stelzer CP, Snell TW (2005) Sexual communication in an ancient species complex: Evolution of the mixis inducing signal in *Brachionus plicatilis* (Rotifer) (in press)
Stross RG, Hill JC (1965) Diapause induction in *Daphnia* requires two stimuli. Science 150:1463–1464
Suatoni E, Vicario S, Rice S, Snell TW, Caccone A (2005) Phylogenetic and biogeographic patterns in the salt water rotifer, *Brachionus plicatilis* (submitted)
Taga ME, Bassler BL (2003) Chemical communication among bacteria. PNAS 100:14549–14554
Wallace RL, Snell TW (2001) Rotifera. In: Thorp JH, Covich AP (eds) Ecology and classification of North American freshwater invertebrates, 2nd edn. Academic, New York
Yoshida T, Jones LE, Ellner SP, Fussmann GF, Hairston NG (2003) Rapid evolution drives ecological dynamics in a predator-prey system. Nature 424:303–306