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Specificity of crowding response that induces sexuality in the rotifer *Brachionus*

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Abstract

Crowding induced the production of mictic (male-producing) females in *Brachionus calyciflorus* from two North American strains and an Australian strain. The specificity of this response to crowding was tested by culturing single individuals of a North American clone in three treatment conditions: a small volume (high density), a large volume (low density), and a large volume with a high density of an Australian clone. The results were consistent and clear in six experiments using different combinations of clones. Crowding low-density individuals of the North American strains with the Australian strain failed to induce them to produce mictic females. The mictic-female response in this treatment was similar to that in the low-density treatment, and both of these responses were significantly lower than that in the high-density treatment. Since the mictic-female response to crowding in *Brachionus* is mediated by a chemical produced by the rotifers themselves, the chemical inducers produced by the Australian and North American strains must be different. Taxonomically specific responses to crowding should increase fitness by assuring that sexual reproduction in the heterogonic life cycle coincides with a high population density of individuals able to mate with one another and, thus, when the production of fertilized resting eggs can be maximized. This would be especially important in plankton communities with diverse rotifer assemblages and multiple congeneric species. Otherwise, a low-density population of a species could be induced to initiate bisexual reproduction by populations of other species, curtailing its potential for population growth via female parthenogenesis and limiting its production of resting eggs in the future.

Changes in zooplankton demographics and reproductive mode may occur at high population densities, and they can be induced by chemicals produced by the population itself. Crowding in laboratory populations of cladocerans and rotifers can affect feeding, growth, age at first reproduction, and fecundity. In many of these cases, the positive or negative effects of crowding are attributable to unidentified chemicals produced by the population (Seitz 1984; Helgen 1987; Matveev 1993; Goser and Ratte 1994; Burns 1995, 2000; Kirk 1998; Yoshinaga et al. 1999; Mitchell and Carvalho 2002). In addition, crowding provides a stimulus for the initiation of bisexual reproduction in both *Daphnia* (Stross and Hill 1965) and *Brachionus* (Gilbert 1963*a*, 2002; Hino and Hirano 1976; Snell and Boyer 1988). The basis for these crowding effects also is unidentified chemicals produced by the population (Hino and Hirano 1976; Hobæk and Larsson 1990; Kleiven et al. 1992; Carmona et al. 1993; Stelzer and Snell 2003).

While considerable attention has been given to demonstrating the chemical nature of crowding effects, less has been devoted to determining the species specificity of crowding responses or crowding chemicals. The chemicals responsible for affecting growth and fecundity in *Daphnia* operate across species (Seitz 1984; Burns 1995, 2000), and water conditioned by *Daphnia carinata* inhibits feeding in conspecifics, *Daphnia lumholtzi*, *Moina micrura*, and *Diaphanosoma unguiculatum* (Matveev 1993).

Three studies have tested the specificity of the crowding stimulus for sexual reproduction in *Daphnia* and *Brachionus*. Male production in *Daphnia magna* was higher in media conditioned by either conspecifics or *Daphnia pulicaria*, but the effect in the one experiment with *D. pulicaria* was not significant ($P = 0.106$; Hobæk and Larsson 1990). Conflicting results have been obtained with *Brachionus*. Early experiments indicated considerable specificity. In *Brachionus calyciflorus*, a population density of four females per milliliter induced the production of mictic (male-producing) females, but only densities of *Brachionus angularis* greater than 100 individuals ml^{-1} induced mictic females in *Brachionus calyciflorus* (Gilbert 1963*a*). In contrast, the specificity of the crowding response in *Brachionus plicatilis* appears to be low. Medium conditioned by conspecifics, and also by the brine shrimp *Artemia salina*, increased the production of mictic females (Carmona et al. 1993).

Understanding the timing of bisexual reproduction in natural species populations of *Brachionus* requires knowing whether the mictic-female response to crowding is sensitive only to the density of its own population or also to that of other, co-occurring populations, perhaps especially congeneric populations. Species of *Brachionus* do commonly cooccur. These species may be morphologically distinct and long recognized or they may be morphologically very similar but genetically differentiated and reproductively isolated by specific mating behavior (Gómez et al. 2002). Of especial note is a series of excellent studies on sympatric sibling species from the *B. plicatilis* complex in a salt marsh in Spain (Gómez and Serra 1995; Gómez et al. 1995; Serra et al. 1998; Ciros-Pérez et al. 2001).

Theoretically, the stimulus for initiation of sexual reproduction in *Brachionus* should be at least species specific. High population density has been viewed as an ecologically appropriate stimulus for this transition because it signals conditions within the population that maximize the production of fertilized resting eggs (Gilbert 1993). Increasing population density permits a higher encounter probability be-

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tween males and young, fertilizable mictic females and also indicates the availability of good food resources for the production of energy-rich resting eggs by fertilized mictic females. The proposed advantages of an association between crowding and sexual reproduction would not be realized if individuals of one species responded to high population densities of other species. A developmental commitment to sexual reproduction at a low population density would curtail population growth via female parthenogenesis and lead to the production of fewer fertilized resting eggs (Serra and King 1999; Gilbert 2002).

The present study examines the specificity of the crowding response in *B. calyciflorus*. The design of the experiments was to use single and mixed cultures of two strains that can be visually distinguished from one another and that produce mictic females in response to crowding. In the first set of experiments, two North American and one Australian strain were cultured singly at a low and a high population density to determine if crowding induced the production of mictic females. In the second set of experiments, a North American strain was cultured at a low and high population density by itself and at a low population density with a high population density of the Australian strain. In this way, the ability of the Australian strain to induce the North American strain to produce mictic females was tested. Another possible, but less natural, approach for testing this cross-induction would be to culture the North American strain in a filtrate of medium conditioned by a high population density of the Australian strain. However, if the crowding chemical was labile, the filtrates could rapidly loose activity. Also, if additional stimuli other than the crowding chemical, such as physical contact, enhanced the induction of mictic females, filtrates alone could provide a weak stimulus.

Methods

All clones of the *B. calyciflorus* strains were initiated from resting eggs. Resting eggs from a Florida strain (University of Florida campus, Gainesville) were purchased from Florida Aqua Farms. Resting eggs from a Georgia strain (Piedmont Park Pond, Atlanta) were kindly provided by Terry W. Snell. Resting eggs from the Australian strain were in dried sediment collected on 28 May 1997 from above the water level of Ryan's 2 billabong near Wodonga, Victoria. The Florida and Georgia strains are phenotypically very similar, and males of each strain will both mate and copulate with females of the other (Gilbert unpubl. data). The Australian strain is distinct from the North American strains in morphology and swimming behavior, and cross-copulation does not occur (Gilbert unpubl. data).

The rotifers were fed *Cryptomonas erosa* var. *reflexa* cultured in modified MBL (Marine Biological Laboratory) medium (Stemberger 1981). In early experiments, the rotifers were cultured in filtered (0.45 μm) water from Storrs Pond (Hanover, New Hampshire) brought to pH $\sim$ 7.5 with 0.5 N NaOH; in later experiments, the rotifers were cultured in the defined MBL medium. Rotifer populations grew similarly well in both media. Rotifer and algal cultures and all experiments were maintained at 20°C (light : dark 16 : 8 h). *Cryptomonas* cell densities were determined with a hemacytometer.

Experiments were conducted using several clones from each strain. Each clone was a population derived from a female hatching from a resting egg, and clone numbers refer to these genetically distinct populations. Different clones were used for two reasons. First, after prolonged culture, some clones were discarded because they became unhealthy or were unlikely to produce mictic females when crowded. Second, the analysis of strain responses would be more robust if based on more than a single clone. The different clones of a strain appeared to be very similar to one another with regard to their responses to crowding, and no attempt was made to look for clonal variation.

To test the effect of population density on the propensity of amictic females to produce mictic daughters, females were cultured individually in large or small volumes of medium. Large volumes were 5 or 15 ml for the North American strains and 40 ml for the Australian strain. A larger volume was used for the latter because they have a lower threshold population density for the production of mictic females, possibly because of their larger size. Mean (1 standard error [SE]) lorica lengths of live, adult amictic females carrying one or more eggs were 167.5 (2.0) μm for the Florida strain (clone 40) and 219.5 (2.3) μm for the Australian strain (clone 20). The small volume used for the high-density treatment was 1.5 ml for all strains. Culture vessels used were the concavities of 24-well tissue-culture plates (Becton Dickinson and Company, Falcon® 3047) for 1.5 ml; 35 $\times$ 10–mm petri dishes (Falcon® 1008) for 5 ml; 60 $\times$ 15–mm petri dishes (VWR Scientific Products) for 15 ml; and 50-ml glass beakers for 40 ml. *Cryptomonas* cell density was 2 $\times$ 10^4 cells ml^{-1} in both treatments of all experiments, except the one with Florida clone 34, in which case cell density was 3 $\times$ 10^4 cells ml^{-1}.

Experiments were initiated by introducing single newborn females into each vessel. These females were collected after a 4- to 7-h period from batches of ovigerous adults originating from cultures maintained at low population densities ($<$2 rotifers ml^{-1}) and high food levels (2–3 $\times$ 10^4 *Cryptomonas erosa* cells ml^{-1}). Every day the females in each treatment were transferred to fresh conditions and their daughters were removed and cultured singly in 0.2 ml medium in concavities of 96-well tissue-culture plates (Falcon® 3077) until they produced eggs and could be typed as amictic or mictic. The latter produce much smaller eggs that develop into diminutive males. Experiments were terminated after 4–5 d. The number of replicate females per treatment varied from 6 to 18 and usually differed across treatments within experiments because some of the initial females turned out to be mictic and were discarded. Details of the experiments are given in Table 1.

Six experiments were conducted to test the ability of the Australian strain to induce the production of mictic females in the North American strains. Each experiment had three treatments. Two treatments were a low and high population density of a North American clone. These were controls to verify that a low density of the clone by itself would induce few mictic females, and that the clone had the potential to produce a high percentage of mictic females if self-crowded.

Table 1. Details of five experiments testing the effect of crowding on mictic-female production in three strains of *Brachionus calyciflorus*: Florida (FL), Georgia (GA), and Australia (AUS). Daily-renewed batch cultures were initiated by placing a single newborn female into a large (5-, 15-, or 40-ml) or small (1.5-ml) volume of either Storrs Pond water (SPW) or MBL medium (MBL) with *Cryptomonas* at 20°C.

Strain and clone	Cultures		Number of replicate females	Mean (1 SE) number of offspring per female
	Medium	Volume (ml)		
FL 14	SPW	15	12	10.1 (1.0)
		1.5	15	10.4 (0.9)
FL 34	MBL	5	6	12.8 (0.3)
		1.5	8	10.8 (1.1)
GA 3	SPW	15	15	9.7 (0.4)
		1.5	15	10.5 (0.5)
AUS 3	MBL	40	14	10.0 (0.4)
		1.5	18	7.2 (0.2)
AUS 11	MBL	40	14	6.7 (0.5)
		1.5	14	7.4 (0.2)

The experimental treatment was a low population density of the North American clone with a high-density population of an Australian clone.

The protocol for these experiments was similar to that used for the previous set of experiments, in which we tested the effect of low and high population density on the production of mictic females in single clones. Details of each of the six experiments are shown in Tables 2 and 3. Initial cell densities of *Cryptomonas* were either 2×10^4 cells ml^{-1} (Experiments 1–3) or 2.5×10^4 cells ml^{-1} (Experiments 4–6). In all three treatments a single, newborn female of the North American target clone was placed into each culture vessel. For the low- and high-density control treatments, culture volumes were 5 or 6 ml and 0.5, 1.0 or 1.5 ml, respectively. For the experimental treatment with a high density of an Australian clone, 4 to 10 Australian females were added to a low-density population of the North American clone. The number and ages of the Australian females varied across experiments, but the number was always at least as high as that shown to induce a high mictic-female response in the previous set of experiments (one female in 1.5 ml). Adult amictic females probably provided an especially strong mictic-female–inducing stimulus, since each would produce several daughters during the course of a day. Every day, the rotifers were transferred to fresh conditions and the initial population structure of the Australian clone was reset.

As in the previous set of experiments, daughters of target females of the North American clone were removed daily and cultured so that they could be classified as amictic or mictic. In the mixed-strain cultures, these could be visually distinguished from Australian females. In addition to being smaller, females of the North American strains swam more rapidly and were less rotund, especially as juveniles. No identification errors were made; all of the juvenile females removed from the mixed-strain cultures later proved to be of the North American clone.

Nonparametric statistical tests were used to compare percentages of mictic daughters in the different treatments of an experiment because there frequently were few mictic females produced in the low population density treatments. The Mann-Whitney *U*-test was used in the first set of experiments with two treatments. The Kruskal–Wallis test, corrected for ties, was used in the second set of experiments with three treatments; pairwise comparisons were made without correction for ties using the formula of Dunn (Zar 1999, p. 224).

Results

In all three strains of *B. calyciflorus*, mictic-female production at the high population density (one female in 1.5 ml) was significantly greater than at the lower one (Fig. 1). The percent mictic daughters produced in the high-density treatment was highest in the two Australian clones—69% and 83%. Visual inspection of *Cryptomonas* abundance after each 24-h period showed little depletion, even in the high-density treatment. The mean number of daughters produced by the females varied across experiments from about 7 to 10 but usually was similar in the two treatments of a given experiment (Table 1).

All six experiments testing the effect of crowding the Florida or Georgia strains with the Australian strain gave consistent results (Fig. 2; Table 4). The overall treatment effect was highly significant, and pairwise comparisons

Table 2. Culture details of six experiments testing the effect of crowding Florida (FL) or Georgia (GA) clones of *Brachionus calyciflorus* (target clones) with Australian (AUS) clones of *B. calyciflorus.* Each experiment has three treatments: low population density (LD) and high population density (HD) of target clone and low population density of target clone with high population density of an Australian clone. One newborn female of target clone initiated each culture. Adults of the Australian clone were amictic females carrying two eggs.

Experiment	Target-clone treatments			AUS-clone treatment		
					Number of individuals	
	Clone	Volume of LD culture (ml)	Volume of HD culture (ml)	Clone	Neonates	Adults
1	FL 11	6	1.5	5	4	0
2	FL 11	6	1.5	5	2	2
3	FL 22	6	1.5	3	0	4
4	FL 34	5	1.0	11	0	4
5	FL 34	5	1.0	11	5	0
6	GA 3	5	0.5	11	0	10

Table 3. Further details of six experiments testing the effect of crowding North American clones of *Brachionus calyciflorus* with Australian clones of *B. calyciflorus.* Treatments are low population density (LD) and high population density (HD) of a North American clone and low population density of the North American clone with a high population density of an Australian clone (AUS). See Table 2 for culture details. Media are Storrs Pond water (SPW) and MBL medium (MBL).

Experiment	Medium	Treatment	Number of replicate females	Offspring per female	
				Mean (1 SE) number	Mean (1 SE) % mictic
1	SPW	LD	10	7.6 (0.4)	4.4 (2.3)
		LD+AUS	9	7.8 (0.6)	15.8 (7.0)
		HD	12	9.8 (0.4)	64.7 (7.2)
2	SPW	LD	5	10.2 (0.7)	11.5 (7.9)
		LD+AUS	5	7.4 (0.5)	16.6 (6.0)
		HD	4	8.5 (0.3)	63.6 (5.5)
3	SPW	LD	16	9.1 (0.6)	17.0 (3.8)
		LD+AUS	11	6.4 (0.5)	27.4 (4.5)
		HD	15	9.3 (0.7)	63.4 (6.3)
4	MBL	LD	18	7.7 (0.2)	4.7 (2.1)
		LD+AUS	12	8.3 (0.3)	4.6 (2.5)
		HD	18	7.8 (0.2)	29.4 (7.6)
5	MBL	LD	13	11.3 (0.4)	1.3 (0.9)
		LD+AUS	13	13.2 (0.3)	2.4 (1.6)
		HD	13	10.7 (0.6)	11.9 (4.0)
6	MBL	LD	5	8.2 (0.7)	6.0 (6.0)
		LD+AUS	5	9.0 (1.5)	1.5 (1.5)
		HD	7	8.3 (0.6)	77.7 (3.9)

showed the same pattern. When cultured by themselves, the North American clones produced significantly higher percentages of mictic daughters in the high-density treatment than in the low-density one (low density vs. high density comparison, Table 4). This result was expected from that of the earlier set of experiments, although two of the Florida clones (11 and 22) had not been tested earlier. When crowded with an Australian clone, the North American clones produced only a low percentage of mictic daughters. This percentage was not significantly different from that in the treatment in which a North American clone was cultured by itself at the same low density (low density vs. Australian comparison, Table 4). Also, this percentage was significantly lower than that in the treatment in which a North American clone was cultured by itself at a high density (Australian vs. high density, Table 4).

Visual inspection of the *Cryptomonas* after each 24-h period showed that the availability of this resource remained high, even in the crowded treatments. The number of daughters produced by the North American females varied across treatments and experiments from about 7 to 13, but there

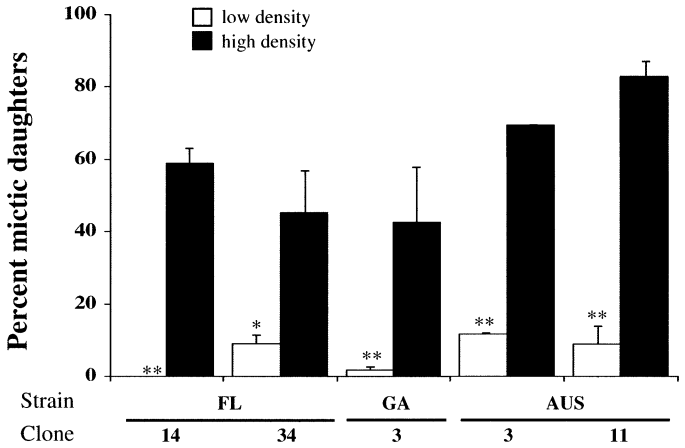

Fig. 1. Crowding induces production of mictic-female daughters in clones from three strains of *Brachionus calyciflorus:* Florida (FL), Georgia (GA), and Australia (AUS). See Table 1 and text for experimental details. Values are means with 1 SE. Means at low and high population densities compared using Mann-Whitney *U*-test; * and ** indicate *P* values <0.05 and <0.0001, respectively.

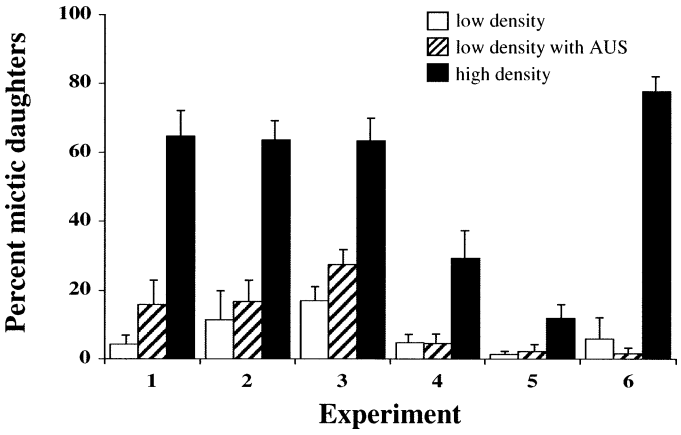

Fig. 2. Six experiments comparing the percent mictic daughters produced by amictic females of Florida (FL) or Georgia (GA) clones of *Brachionus calyciflorus* cultured under three conditions: low population density with or without a high population density of an Australian (AUS) clone of *B. calyciflorus* and high population density. See Tables 2 and 3 for experimental details.

Table 4. Statistical analysis of six experiments comparing the % mictic daughters produced by North American clones of *Brachionus calyciflorus* cultured under three conditions: low population density (LD), low population density with high population density of an Australian clone of *B. calyciflorus* (AUS), and high population density (HD). NS means not significant ($\alpha = 0.05$).

	Kruskal–Wallis test		*P* for Pairwise comparisons		
Experiment	H	*P*	LD vs. AUS	LD vs. HD	AUS vs. HD
1	19.97	<0.0001	NS	<0.001	<0.01
2	9.52	0.0086	NS	<0.001	<0.002
3	21.29	<0.0001	NS	<0.001	<0.001
4	15.41	0.0005	NS	<0.001	<0.001
5	7.75	0.021	NS	<0.005	<0.01
6	13.01	0.0015	NS	<0.001	<0.001

was no tendency for fecundity to be lower in the crowded treatments (Table 3).

Discussion

Crowding induced the production of mictic females in all clones of *B. calyciflorus* from the Florida, Georgia, and Australia strains. Single females cultured in a small volume of medium produced significantly more mictic daughters than those cultured in a larger volume. This result indicates that the mictic-female response does not depend on contact with other individuals in a population and thus is consistent with an earlier study with a Connecticut clone (Gilbert 1963*a*). Crowding also has been shown to induce the production of mictic females in *B. plicatilis* (Hino and Hirano 1976; Snell and Boyer 1988; Stelzer and Snell 2003), *Brachionus angularis, Brachionus variabilis* (Gilbert unpubl. data), *Epiphanes senta,* and *Rhinoglena frontalis* (Thomas Schröder pers. comm.).

The crowding response in *B. calyciflorus* can be attributed to a chemical produced by the rotifers themselves, as suspected by Gilbert (1963*a*). Experimental support for such chemical induction in *B. plicatilis* has been provided by Hino and Hirano (1976), Carmona et al. (1993), and, most notably, Stelzer and Snell (2003). Possible pathways by which a crowding chemical may induce development of mictic females have been detailed elsewhere (Gilbert 1963*a,* 2003).

The major result of the present study is that the crowding response in *B. calyciflorus* has considerable taxonomic specificity. A high population density of the Australian strain—high enough to induce a very high percentage of mictic daughters in that strain—failed to stimulate the production of mictic females in low-density populations of North American strains. The North American clones produced a high percentage of mictic daughters if crowded to the same or lesser extent with their own clonemates. Six separate experiments using four combinations of North American and Australian clones gave the same result.

It is important to emphasize that the Australian strain probably is genetically quite different from the North American strains; it is phenotypically distinct, and also reproductively isolated, from them (Gilbert unpubl. data). A series of cross-induction experiments using more similar strains would be instructive. For example, will the Florida and Georgia strains, which cross-copulate, cross-induce? This question was not addressed using the protocol of the present study because the two strains could not be morphologically distinguished from one another. However, experiments using inductive filtrates of crowded cultures, such as those employed by Stelzer and Snell (2003), could examine cross-induction between phenotypically indistinguishable strains.

An inference from the results of the present study is that the chemical produced by the Australian strain cannot induce production of mictic females in the Florida or Georgia strains and therefore must be different from the one produced by the North American strains. The nature of the crowding chemical that induces the mictic-female response in *Brachionus* has not been investigated, and so the chemical basis for the specificity is unknown.

The specificity of the crowding response demonstrated in the present study is consistent with that reported in an earlier study (Gilbert 1963*a*) showing that *B. angularis* only induced significant production of mictic females in *B. calyciflorus* when its population density exceeded 100 individuals ml^{-1}. No other cross-induction experiments using different strains or species of *Brachionus* have been conducted. However, Carmona et al. (1993) conducted one experiment showing that medium conditioned by the brine shrimp *Artemia* stimulated the production of mictic females in *B. plicatilis.* Thus, it is possible that the inducer may be specific in some species but not in others.

A general consideration of interest is the relationship between cross-induction of mictic females and reproductive isolation. For example, does cross-induction between two clones or strains of *Brachionus* always and only occur as long as they are capable of cross-mating and producing viable hybrids? Or can the crowding chemical produced by one species induce the production of mictic females in another? The results of the present study with the Florida and Australian strains of *B. calyciflorus* certainly indicate that the mictic-female response to crowding could be species specific.

It would be instructive to evaluate the specificity of crowding responses in the *B. plicatilis* complex (Gómez et al. 2002), despite some evidence (Carmona et al. 1993) that the crowding response is not specific. Some of the species in this complex may co-occur and show a seasonal succession as a result of different temperature and salinity niches (Gómez et al. 1997; Serra et al. 1998). They can, however, show considerable overlap in time and even sexual periodicity (Carmona et al. 1995; Ciros-Pérez et al. 2001). Also,

when two of these species are cultured together in the laboratory, they both produce high proportions of mictic females for much of the time (Ciros-Pérez et al. 2002). In both of these natural and laboratory systems, mechanisms controlling the timing and degree of bisexual reproduction in the species cannot be determined unless the extent of cross-induction is known.

The mictic-female response to crowding in rotifers should be more likely to increase fitness if it is taxonomically specific. Specific responses to crowding would assure that production of mictic females in a population occurs only when that population is at a high density and large numbers of individuals are available for cross-mating and production of fertilized resting eggs. This should maximize the number of resting eggs that could be contributed to the sediment egg bank for the initiation of future populations. If the crowding response were less specific, one species could induce mictic females in a small population of another species. Commitment to the production of mictic females in a small population probably would be disadvantageous because few resting eggs would be produced. The probability of encounters between males and mictic females would be low, and producing mictic females reduces the potential for future population growth via female parthenogenesis (Serra and King 1999; Gilbert 2002).

Specific crowding responses for the production of mictic females could evolve in two different ways. Independent evolution of geographically isolated populations could produce differences in the chemical structure of the inducer as well as in other traits. If these populations then became sympatric, cross-induction might not occur. This probably explains the failure of the Australian strain in the present study to induce production of mictic females in the North American strains. Alternatively, if two sympatric populations were unable to cross-fertilize and produce viable hybrids, then natural selection might favor differentiation of the chemical inducers.

Taxonomically specific crowding responses should decrease the coincidence of sexual periods of co-occurring congeneric species. Therefore, they should reduce the potential for matings between species and hybridization. However, if different species were to attain high population densities and initiate sexual reproduction during the same time period, cross-matings and hybridization likely would be prevented by species-specific mating reactions (Gilbert 1963*b*; Snell 1989; Hagiwara et al. 1995).

It is clear that crowding affects the initiation and degree of bisexual reproduction, and hence population dynamics, in some rotifers and cladocerans. It is also clear that this density-dependent response can be mediated, at least in part, by chemicals produced by individuals in the population. However, to fully understand the extent of sexual reproduction in natural species populations, the taxonomic specificity of this crowding response must be known. The present article on the rotifer *B. calyciflorus* is a step in this direction and shows that the response of North American strains cannot be induced by an Australian strain. However, further research must be conducted on naturally co-occurring species and clones to determine the specificity of this response. Future work could also address the identity of the inductive infochemicals and the neurophysiological or developmental mechanisms by which they operate.

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