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Sexual reproduction in *Daphnia pulex* (Crustacea: Cladocera): observations on male mating behaviour and avoidance of inbreeding

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SUMMARY

1. Mating behaviour in *Daphnia* appears to rely on random contact between males and sexual females rather than diffusible pheromones. Males may be able to discriminate sexually receptive females from females in other developmental stages and increase their mating efficiency. Males may also use chemical signals to avoid mating with females from the same clone and avoid the severe inbreeding depression that has been documented for intraclonal mating. The present study used experiments to test for the avoidance of intraclonal mating and assess male mating efficiency in *D. pulex*.
2. Three clones were examined for the avoidance of intraclonal mating by providing males with an opportunity to mate with females of the same or two different clones. The proportion of intraclonal matings did not differ from the proportion of interclonal matings, suggesting that *D. pulex* males do not use kin discrimination to avoid mating with females from the same clone.
3. The proportion of mated females decreased with increasing numbers and density of sexual females when exposed to a single male. This observation suggests that a male spends more time pursuing and copulating with sexually receptive females than non-receptive females and there is insufficient time to mate with all sexual females. The decrease in proportion of females mated could also be the result of sperm depletion in the male. Sperm depletion is unlikely to occur in nature because sexually receptive females are much rarer than in the experimental conditions.

Keywords: clone, *Daphnia*, inbreeding, mating behaviour, sexual reproduction

Introduction

Zooplankton inhabit relatively large bodies of water in relation to their small size and are therefore faced with the difficulty of locating and identifying potential mates during sexual reproduction. Gerritsen (1980) refers to the critical density of a population as being the lowest density that allows a sufficient number of male–female encounters to occur while permitting a population to be sustained by sexual reproduction. When density is lower than this critical value, mating encounters decrease to the point where sexual reproduction alone does not sustain the population. Therefore, adaptations that lower the critical density and increase the probability of a successful encounter between mates are expected to evolve (Gerritsen, 1980; Dusenbery & Snell, 1995). Several evolutionary solutions to the problem of finding and identifying mates have been documented for zooplankton. These include adaptations that increase the distance over which mating encounters can occur and therefore reducing the critical density. The maximum distance at which mate location is effective usually depends on the type of sensory system. Eyesight is poorly developed in zooplankton and it appears that mechanoreception, chemoreception and

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swimming in linear directions (scanning behaviour) may play a more important role than vision in locating potential mates (Gerritsen, 1980).

Diffusible chemicals (sex pheromones), used for mate location, have been described for copepods (Uchima & Murano, 1988; Snell & Morris, 1993; Lonsdale, Frey & Snell, 1998). Male copepods of *Pseudodiaptomus coronatus* Williams and *Eurytemora affinis* Poppe detect the presence of females by using a non-specific chemical signal (Katona, 1973; Snell & Morris, 1993). This signal requires no prior female contact and is also effective for locating females once they have been detected (Katona, 1973). In addition to diffusible pheromones used for locating mates, males of some copepod species have been shown to use contact pheromones to identify developmentally mature conspecific females (Kelly, Snell & Lonsdale, 1998). Thus, chemical communication appears to play a major role in increasing mating efficiency in copepods living at low density (Lonsdale, Frey & Snell, 1998).

In contrast to copepods, rotifers appear to have species-specific contact pheromones on the surface of females rather than diffusible pheromones (Snell & Morris, 1993). Males of *Brachionus plicatilis* O. F. Muller and *Asplanchna brightwelli* Gosse have been shown to initiate a mating response only if they contact an area of the female's corona that releases a chemical (Gilbert, 1963; Aloia & Moretti, 1973). In *B. plicatilis*, this chemical signal has been identified as a surface glycoprotein called mate recognition pheromone that aids in the location and recognition of conspecific females (Rico-Martinez & Snell, 1995). In addition to chemical signals, rotifers have overcome the problem of finding mates at low density through random swimming and by increasing the population density through parthenogenetic (asexual) reproduction prior to sexual reproduction (Gerritsen, 1980; Snell & Garman, 1986; Dusenbery & Snell, 1995).

Cladocera have also had to evolve mechanisms for increasing the probability of encounter between mates at low density. Compared with copepods and rotifers, however, the mating behaviour of cladocera such as *Daphnia* has received much less attention (Snell & Morris, 1993). Similar to rotifers, *Daphnia* have overcome the restrictions imposed on sparse populations of sexually reproducing zooplankton primarily through cyclical parthenogenesis, in which parthenogenetic reproduction increases population density prior to the production of the sexual stages. Cyclical parthenogenesis is characterised by separate parthenogenetic and sexual phases (Hebert, 1978). The parthenogenetic phase consists of females that produce diploid eggs parthenogenetically giving rise to genetically identical female offspring. The environment determines sex in *Daphnia*, with the parthenogenetic phase lasting until crowding, food shortages, and/or changes in day length stimulate the production of males and sexual females. Males mate and fertilise haploid diapausing eggs produced by females entering the sexual phase of the reproductive cycle (Hebert, 1978). Diapausing eggs are enclosed in the ephippium, a protective structure modified from the carapace that the female moults. Diapausing eggs survive unfavourable periods, such as when the habitat freezes or dries. Females do not store sperm and must re-mate prior to the production of successive clutches of sexual eggs.

Despite an increased understanding of factors influencing the switch from parthenogenetic to sexual reproduction (Innes, 1997; Innes & Singleton, 2000), there is very little information on mating behaviour for any *Daphnia* species (Snell & Morris, 1993). Chemical signalling via diffusible sex pheromones does not appear to play a significant role in modifying the mating behaviour of *D. magna* Straus (Crease & Hebert, 1983) and *D. pulicaria* Forbes (Brewer, 1998). However, Brewer (1998) did observe that the average duration of contact between males and females was much longer than the contact between two males, suggesting that males can distinguish between males and females. It has not yet been determined whether males can discriminate between sexual and non-sexual females (Brewer, 1998).

In *Daphnia*, parthenogenetic reproduction combined with environmental sex determination results in clones of genetically identical males and sexual females (Hebert, 1987). Therefore, the switch to sexual reproduction following parthenogenetic reproduction may increase the probability of mating between males and females from the same clone (equivalent to selfing). Previous studies on *Daphnia* have observed the expression of severe inbreeding depression in the progeny from intraclonal mating compared with interclonal mating (Innes, 1989; De Meester, 1993; Innes & Dunbrack, 1993; Deng, 1997). Thus, males given the opportunity to mate in a system containing females of the same clone and different clones may

show a greater proportion of interclonal mating if kin recognition is possible (De Meester & Vanoverbeke, 1999). The probability of intraclonal mating should increase if sexual reproduction were to occur in a small temporary pond following several generations of parthenogenetic reproduction, particularly during an episode of swarming (Young, 1978). Inbreeding avoidance by male and female siblings has recently been documented for the intertidal copepod *Tigriopus californicus* Baker, which also exhibits severe inbreeding depression (Palmer & Edmands, 2000).

The purpose of the present study was to look for evidence of kin recognition, possibly by chemoreception, resulting in the avoidance of intraclonal mating in *D. pulex*. Mate choice by males was examined because of the ease with which mated and unmated females could be distinguished. Although females were not offered a choice of males, females may exert some choice by avoiding the mating advances of a male through escape responses and remain unmated (Brewer, 1998). Observations were also made to determine the mating efficiency of males when exposed to various numbers and proportions of sexual and non-sexual females. Contact pheromones produced by sexual females could possibly increase the frequency of successful mating encounters by reducing the time that males spend trying to copulate with non-sexual females. The ability of males to discriminate between females ready for copulation and females in other physiological states has been described for copepods (Watras, 1983; Uchima, 1985; Kelly *et al*., 1998) and the cladoceran *Moina brachiata* Jurine (Forró, 1997), suggesting that it may be possible for *Daphnia* as well (Carmona & Snell, 1995).

Methods

Sample collection

Samples of *D. pulex* were collected in early spring from a cyclically parthenogenetic population (pond 8A) in southern Ontario and described in previous studies (Innes, 1991, 1997). Samples were obtained using a plankton net (mesh size ~250 μm) towed near the surface of the ponds. The *Daphnia* were placed in plastic jars containing water from the pond in which they were collected and returned to the laboratory.

Laboratory culture

Ninety-six females were isolated into individual 150 mL plastic cups filled with 60 mL of synthetic pond water (zooplankton medium) described by Lynch, Weider & Lampert (1986). The original females and their parthenogenetically produced offspring formed clones of genetically identical individuals. Clones were fed approximately 3 mL of aquarium-cultured algae consisting primarily of *Chorella* sp. once a day, five times a week. Parthenogenetic reproduction was allowed to take place until clones became established before individuals from each clone were taken for analysis of phosphoglucose isomerase (*Pgi*) using cellulose acetate electrophoresis (Hebert & Beaton, 1989). The *Pgi* was chosen because it was known to be polymorphic and highly sensitive to the staining procedure used in cellulose acetate electrophoresis. This sensitivity allows a gel to be run for single diapausing eggs in the event that an ephippium was released prior to the female being removed from the experiment. Recently released eggs have maternally derived enzyme protein and hence exhibit the same *Pgi* genotype as the mother (personal observations). Mated and unmated ephippial females could be distinguished because mated females release one or two eggs into the ephippium while unmated females release none. Stock jars of each clone were maintained under short day photoperiod (8 : 16, L : D) and 15 °C in order to stimulate the production of males and sexual females.

To confirm that the pre-ephippial females used in the experiments had not been previously mated by males in the stock jar, 80 pre-ephippial females with no sign of an ephippium were isolated from stock jars of six clones containing a mixture of males and females. One day later, females that had produced an ephippium were checked for eggs being deposited. Thirty-two of the 80 pre-ephippial females isolated from clones containing a mixture of males and females produced an ephippium. None of the ephippial females released eggs showing that it was safe to assume that pre-ephippial females had not been mated prior to the beginning of the experiments.

Mature males were identified by the presence of a flattened and spread ventral carapace, pronounced setae along the anterior end of the ventral margin, and erect antennules each with a prominent flagellum.

These secondary sex characteristics were associated with the presence of sperm cells observed in the testes under a light microscope (400×). Only mature males, as identified by the secondary sex characteristics, were capable of mating with females as indicated by the release of eggs into the ephippium. Sexual females exposed to males lacking the secondary sex characteristics failed to release diapausing eggs into the ephippium (Winsor, 1997).

Avoidance of intraclonal mating

Forty jars were filled with 600 mL (31 jars), 1200 mL (two jars), 1400 mL (two jars) or 2000 mL (five jars) of conditioned zooplankton medium. This medium consisted of a 50 : 50 mixture of fresh zooplankton medium and zooplankton medium filtered from stock cultures that is believed to contain factors beneficial to the growth of *Daphnia*.

Ten females not carrying a parthenogenetic brood, from each of three clones differing in their *Pgi* genotype, were added to each jar. The females used in the experiments were approximately the same size and were most likely to be pre-ephippial, as indicated by the presence of two well-developed, greenish-brown ovaries on each side of the digestive tract. These females showed no sign of the deposition of pigment associated with the initial development of the ephippium. Females are only receptive prior to the formation of an ephippium, and therefore it was not possible to start the experiments with a known number of sexual females. As a consequence various numbers of the initial 30 females in each experiment were sexually receptive. One newly matured male from the same clone as one of the pre-ephippial females was added to the jar to start the experiment. The time the males were added was recorded and the jars were placed in a short day (8 : 16, L : D) incubator at 15 °C for 20 h. After this period, the male was removed from the jar and the number of ephippial females, as well as the proportion mated as indicated by eggs in the ephippium, was recorded. The *Pgi* genotype was used to determine clone membership for each ephippial female.

The number of matings between males and ephippial females of the same or a different clone were tallied and 2 × 2 contingency tables constructed (see Table 1). Sample sizes for the individual mate-choice experiments were too small to be statistically reliable because of the small number of females that were stimulated to become sexual in each of the experiments. Therefore, tests were carried out on the total of the individual G statistics as well as for the data pooled within each of the different volumes and for the data pooled for all volumes (Sokal & Rohlf, 1981).

Male mating efficiency

Male mating efficiency was assessed by examining the association between the density of ephippial females, the number of ephippial females and the proportion mated using the data from the avoidance of intraclonal mating experiments. In addition, 17 jars were filled with 1400 mL of zooplankton medium and 30 pre-ephippial females of assorted clones prior to one (eight jars) or two (nine jars) mature males of a different clone were added. All jars were placed in an incubator under short day, 15 °C conditions for 20 h. After incubation, males were removed and the number of ephippial females along with the proportion of ephippial females mated was recorded. Spearman's rank correlation (r_s) was used to determine the association between density of ephippial females, number of ephippial females and the proportion mated. Differences in the proportion mated between the experiments with one or two males were tested using a Wilcoxon two-sample test.

Results

Avoidance of intraclonal mating

Seventeen of the 40 experimental jars produced sufficient numbers of mated and unmated females to test for differences in proportion of females mated between the same or different clones (Table 1). No significant differences in the proportion of mated females were observed between males and females from the same clone compared with males and females from different clones based on the total G-values for the individual experiments within each volume (Table 1). These results indicate that there was no consistent trend in the individual experiments that would suggest an avoidance of intraclonal mating. For the 17 experiments, the average proportion of mated females was 0.545 (SE 0.075), when males and females

were from different clones, and 0.548 (SE 0.076) when males and females were from the same clone. Pooling the data for each volume and for all of the experiments (Table 2) also showed no significant difference ($P > 0.05$) in the proportion mated for interclonal (0.439) and intraclonal (0.459) mating.

Table 1 Number of mated and unmated ephippial females, and percentage mated for males and females from the same or different clones and the clone used for the male in each experiment. G (d.f. = 1) -statistic for individual experiments and total G-values (all $P > 0.05$) for each volume. The significance of the individual *G*-tests was not determined because of small sample size

Volume	Mating	Mated	Unmated	Mated (%)	Clone	G-value	Total G (d.f.)
600 mL	Different	1	1	50	8A1-3	2.634	10.553 (8)
	Same	4	0	100			
	Different	6	0	100	8A1-3	3.255	
	Same	1	1	50			
	Different	2	1	67	8A1-1	0.583	
	Same	7	1	88			
	Different	6	1	86	8A1-1	0.537	
	Same	2	0	100			
	Different	1	1	50	8A1-1	1.726	
	Same	2	0	100			
	Different	1	0	100	8A1-1	1.726	
	Same	1	2	67			
	Different	1	3	25	8A1-1	0.058	
	Same	1	2	67			
	Different	3	1	75	8A1-3	0.032	
	Same	4	1	80			
1200 mL	Different	4	7	36	8A3-17	0.118	4.769 (2)
	Same	2	5	29			
	Different	8	2	80	8A3-17	4.651	
	Same	2	5	29			
1400 mL	Different	4	9	31	8A1-3	0.002	0.074 (2)
	Same	3	7	30			
	Different	4	6	40	8A1-3	0.072	
	Same	2	4	33			
2000 mL	Different	5	13	28	8A1-1	0.013	9.737 (5)
	Same	1	3	25			
	Different	5	11	31	8A1-1	0.238	
	Same	2	7	22			
	Different	5	13	28	8A1-3	0.015	
	Same	3	7	30			
	Different	5	0	100	8A1-1	2.969	
	Same	1	1	50			
	Different	0	9	0	8A1-3	6.502	
	Same	1	0	100			

Table 2 Total number of mated and unmated females from Table 1 for females from the same or a different clone as the male pooled for each volume and pooled for all volumes. G tests for independence (all $P > 0.05$)

Volume	Mating	Mated	Unmated	Mated (%)	G (d.f. = 1)
600 mL	Different	21	8	72.4	0.09
	Same	22	7	75.9	
1200 mL	Different	12	9	57.1	2.71
	Same	4	10	28.6	
1400 mL	Different	8	15	34.8	0.05
	Same	5	11	31.3	
2000 mL	Different	20	46	30.3	0.002
	Same	8	18	30.8	
Pooled	Different	61	78	43.9	0.08
	Same	39	46	45.9	

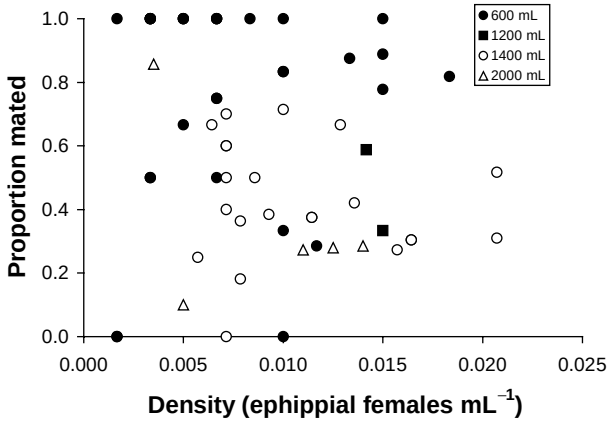

Fig. 1 Proportion of mated females as a function of the density of ephippial females for all experiments. Different symbols represent the different volumes for each experiment. The 0.00 proportion mated values were omitted from the correlation analysis.

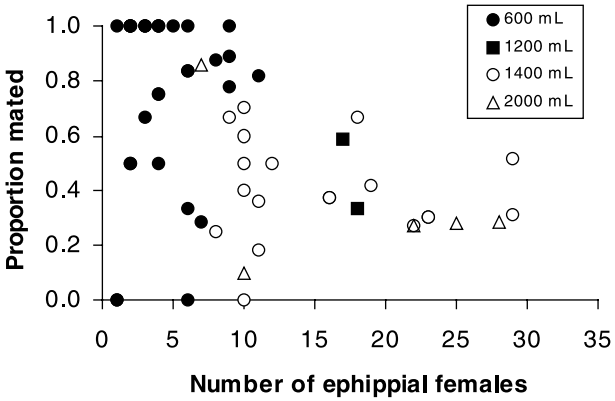

Fig. 2 Proportion of mated females as a function of the number of ephippial females for all experiments. Different symbols represent the different volumes for each experiment. The 0.00 proportion mated values were omitted from the correlation analysis.

Male mating efficiency

A large variation in the proportion of mated females was observed among the experiments (Figs 1 and 2). Four of the 57 experiments resulted in no mated females, suggesting that the males used in these experiments were likely to have been infertile. There was a significant negative relationship ($r_s = –0.408$, $P = 0.0025$) between the proportion of ephippial females mated and the density of ephippial females when the data from the different volumes were plotted together (Fig. 1). A significant negative correlation ($r_s = –0.688$, $P = 0.0001$) was also observed

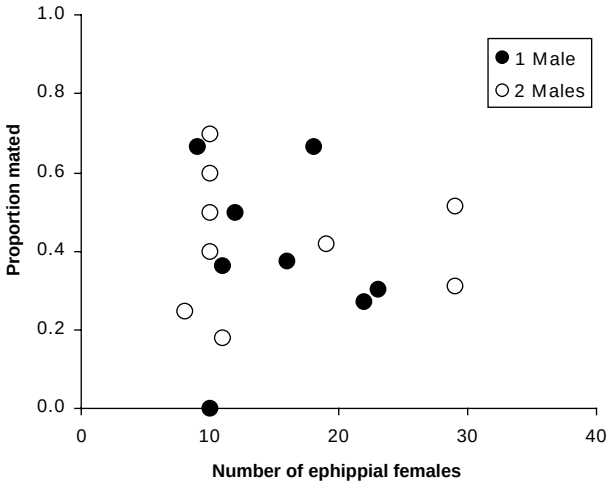

Fig. 3 Proportion of mated females as a function of the number of ephippial females for the 1400 mL experiments involving one or two males. The 0.00 proportion mated value was omitted from the correlation analysis.

between the number of ephippial females and the proportion mated (Fig. 2). However, no significant relationship was observed between the proportion of ephippial females mated and the number of ephippial females for the 1400 mL volume experiments involving one ($r_s = –0.577$, $P = 0.18$) or two ($r_s = –0.43$, $P = 0.91$) males, nor when the data were pooled ($r_s = –0.203$, $P = 0.45$) (Fig. 3). The average proportion of ephippial females mated for one male (0.450 ± 0.062 SE) was not significantly ($P > 0.05$) different from the experiments with two males (0.431 ± 0.59), omitting the experiment with no mated females.

Discussion

Test for kin recognition

Daphnia pulex live in small, temporary ponds where there is the possibility that males and sexual females from the same clone will encounter each other and mate. While genetic studies did not detect any evidence for inbreeding occurring in the population of *D. pulex* that was sampled for the present study (Innes, 1991), the laboratory experiments described here found that kin-recognition does not appear to play a role in avoiding the inbreeding that would result from within-clone mating. All of the experiments showed no significant difference in the proportion of successful matings between males and females from the same or different clones. Data for the

individual experiments were pooled together to increase the probability of detecting slight differences, although it appears that direct avoidance of inbreeding was not occurring or was undetectable because of the small sample sizes for the particular clones tested increasing the probability of type II errors.

As with many other organisms, female reproductive success in *Daphnia* is limited by the number of sexual eggs that she can produce, rather than by her access to mates. Natural populations of *D. pulex* show an excess of males over sexual females and few, if any, sexual females remain unmated (Innes, 1997). Male reproductive success is limited by access to females and results in a situation favouring the evolution of mate choice by females. Mate choice by *Daphnia* females is primarily limited to escape responses (Brewer, 1998), although there may also be a role for choice at the gamete level after copulation with multiple males. In the present experiments only males were tested for any evidence of avoidance of intraclonal mating. Given the confines of the jar and the length of the experiment, any female escape response may be ineffective in avoiding a male from the same clone, particularly as a male only needs about 15 s to effect copulation (Brewer, 1998). The female escape response may, however, be an effective mechanism for avoiding a male from the same clone when males from other clones are present. For example, escape responses by females appeared to be effective in reducing the probability of inbred mating in the copepod *T. califonicus* when females were given a choice between unrelated males and male siblings (Palmer & Edmands, 2000). Observations on female choice are possible in copepods because males clasp females and mate guard for several days. Female choice experiments would be very difficult to carry out in *Daphnia* using behavioural observations alone because no mate guarding occurs and copulation is over in a few seconds. However, tests for choice by females will be possible with recently developed microsatellite genetic markers (J.K. Colbourne & D.J. Innes, unpublished data). Genetic markers can be used to determine the paternity of diapausing eggs and thus to test if female *Daphnia* avoid mating with males from the same clone when given a choice that includes males from different clones.

While kin discrimination was not apparent, other strategies for the avoidance of inbreeding probably occur in *Daphnia*. One mechanism that may reduce the probability of inbreeding was described by Jarne & Charlesworth (1993) and involves aquatic animals and wind-pollinated plants diluting their gametes into the environment. Although *Daphnia* do not release gametes into the environment for external fertilisation, individuals from a parthenogenetic brood may disperse some distance from their site of release within the pond to obtain a similar result. Species that disperse from their site of birth increase the probability of avoiding kin and reduce the importance of kin recognition in the avoidance of inbreeding. The ability to recognise kin is better developed in organisms that have dispersal patterns making it likely that close relatives encounter each other as sexually mature adults (Pusey & Wolf, 1996).

The avoidance of inbreeding through dispersal can be enhanced by producing male and female offspring or gametes at different times. In some species of protandrous and protogynous plants, inbreeding can be avoided through desynchronisation of the male and female phases (Jarne & Charlesworth, 1993) and may be paralleled in *D. pulex* by the release of same-sex parthenogenetic broods. In *Daphnia*, parthenogenetic females produce broods that are generally all male or all female (Banta, 1939; Innes, 1997). Barker & Hebert (1986) observed that successive broods in *D. magna* are released approximately 72 h apart (at 20 °C). This delay, combined with the time required to become sexually mature, may effectively ensure that males and females from the same clone have a very low probability of encountering each other. Our own observations indicate that it takes about 8 days for males to reach sexual maturity under laboratory conditions (Winsor, 1997), providing a window of time for males and females of the same clone to disperse to different areas of the same pond. In addition, the large number of unrelated individuals present in the pond during periods of sexual reproduction (Innes, 1991) would further make the probability of intraclonal mating negligible.

Dispersal indirectly reduces the probability of intraclonal mating, although other aspects of sexual reproduction may have evolved in direct response to inbreeding avoidance in *Daphnia*, particularly because of the high levels of inbreeding depression that have been observed (Innes, 1989; De Meester, 1993; Innes & Dunbrack, 1993; Deng, 1997). For example, De Meester & Vanoverbeke (1999) suggested that uncoupling of investment in males and sexual females by the

same clone in natural populations of *D. magna* is important in avoiding intraclonal mating, particularly in populations dominated by only a few clones. In most cases, males had genotypes different from sexual females, suggesting that each clone produces males and sexual females in response to slightly different environmental cues. Similarly, Innes & Dunbrack (1993) proposed that the specialisation of clones of *D. pulex* as male- or female-producing is an evolutionary response to the negative effects of inbreeding.

Male mating efficiency

In cyclically parthenogenetic species, the switch from parthenogenetic to sexual reproduction is associated with increased population density and may be an adaptation to increase mating encounters by the sexual stages (Muenchow, 1978; Gerritsen, 1980). Because *Daphnia* and rotifers do not appear to use chemical cues to detect mates at a distance, an encounter between mates is likely to be a random process. Following the hatch of diapausing eggs in the spring, the probability of encounters between mates would initially be below the critical density required to maintain a population by sexual reproduction alone (Gerritsen, 1980). The switch to males and sexual females should only occur when the population density is sufficient to ensure encounters with mates. In addition, male *Daphnia* appear to use a variety of swimming behaviours to increase the probability of encountering potential mates (Brewer, 1998). Even with an increased probability of encounters between potential mates some sort of mating behaviour and mate recognition is required for mating to occur. Once an encounter has been made, male *Daphnia* may rely on hydromechanical disturbances to detect potential mates rather than diffusible chemicals (Crease & Hebert, 1983; Brewer, 1998). After contact it appears that males can discriminate between females and other males because much less time is spent in contact with males compared with females (Brewer, 1998). However, no information is currently available to determine if males can discriminate between sexual and non-sexual females.

If the 20 h used in the present experiments were insufficient for a male to encounter all sexually receptive females at random we would expect that experiments with the lowest density of sexual females to show the greatest number of 0.0 proportion mated. However, the mating success of males did not appear to be limited by the time required to encounter sexual females even at the lowest density. Only four of the 57 experiments showed 0.0% mating success (probably because of infertile males) and several examples (14 of 31) of 100% mating success were recorded for a range of densities of sexual females in the 600 mL experiments including the lowest densities. Thus, it is reasonable to conclude that the experimental conditions were sufficient to allow a single male to encounter randomly each of the 30 females at least once and mate with any of the sexual females encountered.

If a male spends the same amount of time pursuing and contacting a sexually receptive female as with a non-receptive female, and attempts to fertilise any female he contacts during the 20-h period, then mating success should not decrease with increasing numbers of sexual females present. However, mating success did show a significant decrease with increasing numbers and density of sexual females. These results suggest that either a male spends more time pursuing and copulating with sexually receptive females, and there is insufficient time to mate with all sexual females, or the male may have depleted all of his sperm leaving some sexual females unmated. Observations by Brewer (1998) suggested that a successful copulation might only require less than a minute of contact between males and females. Although his experiments did not specifically measure the contact time between males and sexually receptive females, it is likely that copulation does not require more than a few minutes. A short contact time required for copulation combined with sufficient time to encounter all receptive females means that sperm limitation could also explain the reduction in proportion mated with increasing numbers of sexual females. This hypothesis could be tested by using the same male in experiments with successive batches of receptive females. The hypothesis would be rejected if success batches of females continued to become mated.

If either time or sperm limits the ability of a single male to mate successfully with all receptive females then increasing the number of males should increase the proportion of mated females. However, no increase in proportion of mated females was observed in the experiments comparing one versus two males. It is not clear why no effect was observed by adding a

second male. Part of the reason might be because the restricted-area spiral that both males would swim in an attempt to make contact results in the males spending more time pursuing each other rather than females (Brewer, 1998). However, Brewer (1998) found that the male–male contact time was much shorter than the male–female contact time. Experiments to compare male time budgets for one male versus two or more males would be useful to determine if male–male interference prevents an increase in the proportion of females mated.

A lot of variation was observed in the proportion of ephippial females that were mated, particularly for the experiments with 10 or less ephippial females. This could be because of some females being past or almost past the receptive period when they were included in the experiments. This does not seem likely because they were selected from stock cultures with many mature males so that all of the ephippial females were likely to have become receptive sometime during the course of each experiment. The receptive period of a female is unknown but may be as little as a few hours. Therefore, some females may have remained unmated simply because they were no longer receptive by the time the male made contact. In addition, some of the observed variation could be the result of variation in male fertility or mating ability such as proficiency at pursuing and clasping with females. An increased understanding of mating in *Daphnia* would benefit from further investigations on the receptive period of females and variation in male mating ability.

Our results demonstrate that the mating behaviour in *D. pulex* is complex. Additional direct (Brewer, 1998) and indirect observations are required in order to bring our understanding of mating behaviour in *Daphnia* up to that for copepods and rotifers. More detailed studies on *Daphnia* mating will also contribute to a better understanding of how various zooplankton species have solved the problem of sexual reproduction and mate location. Studies to date on mate choice in zooplankton have been limited primarily to the mate-recognition systems required to identify conspecific mates (Snell & Morris, 1993; Lonsdale *et al*., 1998). Some observations have documented male mating behaviours that can identify the reproductive status of females and select receptive over non-receptive females (Watras, 1983; Uchima, 1985; Forró, 1997; Kelly *et al*., 1998). However, an area that has been virtually unexplored for zooplankton species is sexual selection and mate choice. Palmer & Edmands (2000) were able to use direct observations to show that females of the copepod *T. californicus* Baker avoided mating with male siblings whereas no mate choice was evident between males from the same or different populations. Recently developed methods for using genetic markers in offspring to identify the parents will greatly expand the opportunity to assess variation in mating success among males and females of various zooplankton species, including *Daphnia*.

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