Oecologia (1997) 111:53–60 © Springer-Verlag 1997

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Sexual reproduction of *Daphnia pulex* in a temporary habitat

Received: 19 February 1996 / Accepted: 20 January 1997

Abstract Species of *Daphnia* (Crustacea: Cladocera) typically reproduce by cyclical parthenogenesis, in which a period of all-female parthenogenetic reproduction is followed by sexual reproduction. Sex in *Daphnia* is determined by the environment, with factors such as temperature, photoperiod and crowding stimulating the production of males and sexual females. Previous studies on *Daphnia pulex* from temporary pond habitats demonstrated the coexistence of male-producing and non-male-producing (NMP) females, as determined under crowding in the laboratory. A strong genetic component to this sex allocation variation suggested that sex expression in *D. pulex* is better described as a result of genotype-environment interaction. The present study examined the switch from parthenogenetic to sexual reproduction in two temporary-pond populations of *D. pulex*. Both populations showed a very early investment in sexual reproduction, independent of population density, by producing males very soon after the populations were reestablished from resting eggs in the early spring. Approximately 40% of the initial broods were male. Additional evidence for gender specialization was obtained by observing the sex of two or three successive broods for 85 individual females. Fifty-eight females produced successive broods of females, 13 females produced successive broods of males and 14 females produced successive broods which included both male and female broods. Females that produced successive female broods under natural conditions included a higher frequency of NMP females compared to a random sample of females, confirming the existence of NMP females. Sexual females were observed in both populations after the first appearence of males, suggesting that the presence of males may stimulate the production of sexual females. For *D. pulex* populations in a temporary environment, there appears to be an increased emphasis on sexual reproduction and a decreased influence of the environment on sex determination, compared to *Daphnia* populations in more permanent habitats.

Key words Sex allocation · *Daphnia pulex* · Parthenogenesis · Sex determination · Reproduction

Introduction

Genotypic sex determination occurs when sex is determined by an individual's genotype, and is most pronounced in organisms with sex chromosomes (Bull 1983). Sex is determined at conception. In organisms with environmental sex determination (ESD), sex is determined during embryonic development in response to particular environmental conditions (Bull 1983; Adams et al. 1987; Korpelainen 1990). An embryo has the potential to be either sex, and some feature of the environment switches on genes that determine which sex will develop.

Although sex in organisms with ESD is primarily controlled by the environment, this does not preclude genetic variation for the degree of ESD (Bull 1983; Naylor et al. 1988; Lagomarsino and Conover 1993). Studies on genetic variation for sex determination in organisms with ESD are useful for testing theories on the evolution and maintenance of ESD (Bull 1983; Blackmore and Charnov 1989; Conover et al. 1992; Watt and Adams 1994). ESD is expected to evolve when the environment experienced by an offspring affects the fitness of each sex differently and offspring cannot choose their environment (Charnov and Bull 1977).

Species of the planktonic crustacean *Daphnia* (Crustacea: Cladocera) appear to have ESD (Hebert 1978; Korpelainen 1990). Reproduction is by cyclical parthenogenesis in which there is a parthenogenetic (asexual) phase and a sexual phase. Parthenogenetic reproduction produces broods of genetically identical female offspring under one set of conditions, usually associated with low population density and high food availability (Hebert

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1978). The switch to sexual reproduction appears to be related to a deterioration in the environment due to increased population density and reduced food (Berg 1931, 1934; Banta 1939; Barker and Hebert 1986; Innes and Dunbrack 1993; Innes and Singleton 1994). Other environmental factors such as changes in temperature and photoperiod can also influence the switch from asexual to sexual reproduction (Stross 1969; Ferrari and Hebert 1982; Carvalho and Hughes 1983; Korpelainen 1986, 1989; Hobaek and Larsson 1990; Kleiven et al. 1992; Spaak 1995; Deng 1996). During sexual reproduction, females produce broods of diploid males parthenogenetically and sexual females produce haploid resting eggs that are fertilized by males during mating (Hebert 1978). The sexually produced resting eggs are protected by the ephippium (modified carapace) and are resistant to freezing and desiccation. Populations in temporary habitats, such as small ponds, depend on the production of resting eggs to persist from one year to the next. The resistant resting eggs are also the primary dispersal stage for *Daphnia* (Hebert 1978).

The life history of *Daphnia* appears to satisfy the conditions required for the evolution of ESD (Gerritsen 1980). During the spring, when resting eggs hatch, population density is low. Males and sexual females produced at this time would have a low probability of encountering each other, resulting in a low mating success. However, several generations of all-female parthenogenetic reproduction greatly increases population density, increasing the encounter rate and probability of mating between males and sexual females. Thus, it is advantageous for parthenogenetic females to be produced when density is low and males and sexual females when density is high (Gerritsen 1980).

Most of the information on life history variation in *Daphnia* has been concerned with the parthenogenetic phase of the life cycle (Lynch 1980; Taylor and Gabriel 1993), probably because most studies have been on northern lake populations where sexual reproduction is usually confined to a brief period in the autumn (Frey 1982). However, Threlkeld (1979) noted that sexual reproduction (production of ephippial females) in the spring of a lake population of *Daphnia pulicaria* was associated with the regular decline of the population during midsummer. Recently, more attention has been given to factors controlling sex expression and the evidence for ESD in species of *Daphnia*, especially for species inhabiting small ponds, has been assessed more critically (Hobaek and Larsson 1990; Kleiven et al. 1992; Korpelainen 1992; Yampolsky 1992; Innes and Dunbrack 1993; Innes and Singleton 1994; Deng 1996). For example, it has been suggested that environmental factors elicit a “sex ratio” response in which individual females switch from producing all-female broods to alternating male and female broods, resulting in an equal investment in male and female function (Barker and Hebert 1986; Hobaek and Larsson 1990; Kleiven et al. 1992). Broods with a mixture of males and females are usually rare, suggesting that the mechanism of sex determination affects all individuals within a brood equally (Barker and Hebert 1986; Kleiven et al. 1992). Since sexual reproduction is required to produce the resistant resting eggs of cyclically parthenogenetic *Daphnia*, sexual reproduction is a critical phase in their life cycle in temporary-pond habitats and thus warrants closer examination.

Both obligately and cyclically parthenogenetic populations of *Daphnia pulex* occur in small, temporary ponds in southern Ontario, but rarely coexist in the same pond (Hebert et al. 1988). Obligate parthenogens produce resting eggs by ameiotic parthenogenesis and thus sex allocation is not part of this life cycle. Temporary-pond habitats in this area support large populations of cyclically parthenogenetic *D. pulex* for about 6–8 weeks in the spring before drying up (Innes 1991). Recent observations have suggested that the switch to male production in these populations may not be strictly determined by the environment (Innes and Dunbrack 1993; Innes and Singleton 1994). The pattern of sex allocation, as determined under crowding in the laboratory, revealed the presence of females that produce both males and females (male producing, MP) and females that only produce females (non-male producing, NMP). A genetic analysis showed that sex allocation, with respect to the presence or absence of male production, had a large genetic component (Innes and Dunbrack 1993). Thus, an unpredictable environment may select for more genotypic control of sex determination in *D. pulex*. Genetic variation for sex allocation has also been observed in *Daphnia magna* (Yampolsky 1992), *D. pulicaria* (Deng 1996) and other studies of *D. pulex* (Ruvinsky et al. 1986; Larsson 1991). The occurrence of genetically based variation in sex allocation suggests that some individuals may show an early investment in sexual reproduction in these temporary habitats.

The purpose of the present study was to sample populations of *D. pulex* soon after hatching of resting eggs and monitor the switch from parthenogenetic to sexual reproduction by observing the occurrence of males and sexual females. In addition, the allocation to male and female progeny was determined for individual females to confirm the presence of NMP females under more natural conditions.

Materials and methods

Two ponds (8A, 8B) near Port Rowan in southwestern Ontario (see Innes 1991 for locations) were sampled on April 22, 1991. Both ponds are surrounded by trees in small woodlots and are shaded once the trees are in leaf. Each pond was less than 1.0 m deep and the area of 8B (ca. 100 m^2) was approximately twice that of 8A (ca. 50 m^2). Samples were collected using a plankton net (mesh size 250 μm) towed horizontally just below the water surface. *D. pulex* populations were detected in both ponds on this date, but on a subsequent visit on June 15, 1991, no *D. pulex* were observed. The sex ratio of each sample was determined and a sample of females carrying broods (8A, $n = 128$; 8B, $n = 96$) was placed in individual containers with 60 ml of zooplankton medium (Lynch et al. 1986). Individual clones were fed aquarium-cultured algae (primarily

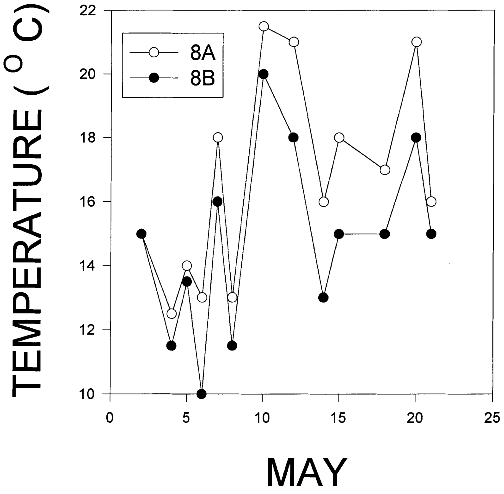

Fig. 1 Temperature for ponds 8A and 8B sampled during May 1992

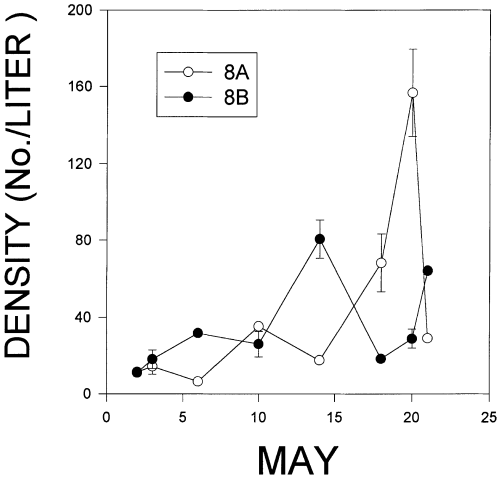

Fig. 2 Population density (mean and SE for two plankton tows) of *Daphnia pulex* for ponds 8A and 8B during May 1992

Scenedesmus and *Ankistrodesmus*) and monitored for male production under high population density as previously described (Innes and Dunbrack 1993).

Density and sex ratio

The two ponds (8A and 8B) were more intensively sampled during a 3-week period in May 1992. Water temperature was measured (a few centimeters below the surface) every 2 days. Both ponds showed large, correlated fluctuations in temperature with a general increase in temperature over the study period (Fig. 1). Each pond was sampled using a plankton net towed horizontally across the pond just below the water surface. Each tow sampled approximately 150 l. Two tows were collected from each pond on each sampling date. *Daphnia* from the tows were preserved in 50 or 100 ml of 95% ethanol depending on the density of individuals in the sample. Subsamples (0.5 or 2.0 ml) were taken from the total sample to estimate the density of males, females with and without broods, and females carrying ephippia. The size of each individual was also measured using an ocular micrometer. Variation in the frequency of males and ephippial females over sampling dates was tested using a one-way ANOVA on the arcsine-square root-transformed proportion (Sokal and Rohlf 1981).

Brood sex

Females carrying a brood were sampled from each pond five times during the 3-week period. Individuals were isolated into test tubes with 10 ml of pond water (filtered to remove zooplankton) and held at room temperature (ca. 20°C) until brood release. The number of males and females was determined for each brood or in a subsample of 50 individuals in the case of brood sizes > 50. On one occasion (May 14) information was collected on the sex composition of a second and third brood produced by individual females in the test tubes. The Heterogeneity of the proportion of male broods over sampling dates was tested using a R × C test (Sokal and Rohlf 1981). A one-way ANOVA tested for variation in brood size among female, male and mixed-sexed broods.

Field cages

Small enclosures (cages) were constructed from 10-ml plastic test tubes covered with 120 μm mesh on the bottom and top of the tube. Cages were anchored in a test tube rack that was submerged just above the bottom of the pond. Individual females carrying a brood were placed in each cage for a total of 36 cages in each of ponds 8A and 8B. Cages were deployed on May 3 and brood size and sex were determined for each female until May 18 (8A) and May 20 (8B). Cages were checked daily and any dead or ephippial females replaced with females carrying a brood.

Sex of laboratory-produced broods

Ninety-six adult females, each carrying a brood, were collected from each of ponds 8A and 8B on May 18 and were raised individually in 100-ml plastic beakers with 60 ml of zooplankton medium (Lynch et al. 1986). Cultures were fed aquarium-cultured algae and a high density of individuals was produced in each culture after 2 weeks of parthenogenetic reproduction. Each clone was monitored for male production under high-density conditions for several weeks. Females that had produced two successive broods of females in cages under natural pond conditions were also monitored for brood sex under crowded conditions in the laboratory as described above. A log-linear analysis of a three-way table (NMP vs MP, 1991 vs 1992, Pond 8A vs Pond 8B) was used to test for independence of the three factors (Sokal and Rohlf 1981).

Results

Density and sex ratio

For the 1992 samples, both ponds 8A and 8B showed an increase in density relative to the initial sample taken on May 2, with large fluctuations in density thereafter (Fig. 2). The proportion of males varied significantly (pond 8A: $F_{7,6} = 7.42$, $P < 0.02$; 8B: $F_{7,6} = 21.01$, $P < 0.01$) among the sampling dates for both ponds (Fig. 3a). Pond 8A showed a higher initial proportion of males then pond 8B and this difference was maintained until near the end of the 3-week period (Fig. 3a). Sex ratios for samples taken early in the previous spring (April 22, 1991) showed a similar difference in proportion of males between the two ponds (Pond 8A: 29.8% male, $n = 500$; 8B: 6.3% male, $n = 269$).

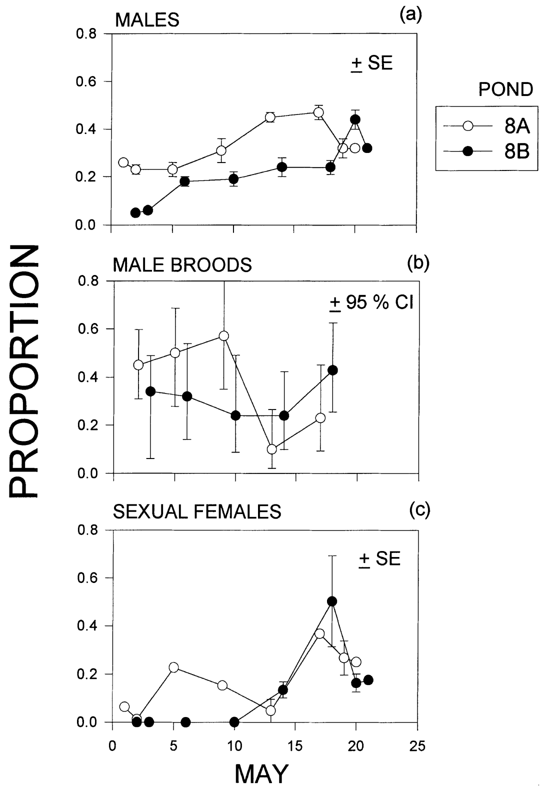

Fig. 3a–c Samples of ponds 8A and 8B to determine the occurrence of sexual individuals of *D. pulex*. Pond 8A values are plotted one day earlier than pond 8B for clarity. **a** Proportion of male individuals. The sample size for each date is the sum of the samples sizes given in Figs. 4 and 5. **b** Proportion of male broods. Sample sizes for each date are given in Table 1. **c** Proportion of sexual (ephippial) females among females > 1.14 mm. Sample sizes for each date are given in Fig. 4

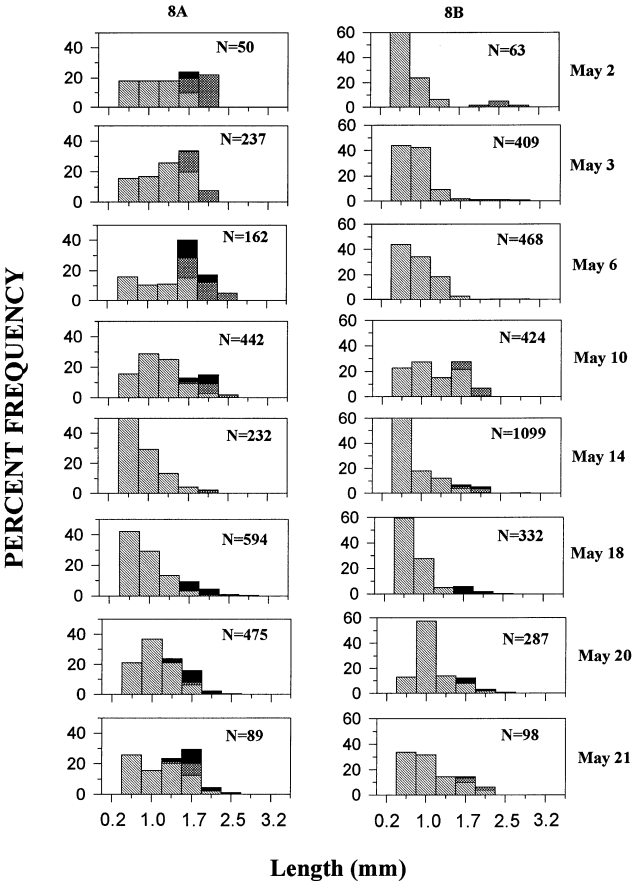

Fig. 4 Length frequency distributions for females without broods (*diagonal-hatched bars*), females with broods (*cross-hatched bars*) and sexual females (*solid bars*) for ponds 8A and 8B sampled on eight dates in May 1992

During the first week (May 2–10, 1992), samples from pond 8A showed a size distribution of females, with the larger size classes carrying broods of parthenogenetic eggs (Fig. 4, Table 1). The size structure in the later samples (May 14–21) was dominated by small females and a smaller proportion of the larger females carried parthenogenetic broods (Fig. 4, Table 1). A small proportion of ephippial females was observed throughout the sampling period (Figs. 3c, 4). On all days but one (May 10) in the study period, pond 8B was dominated by small females, and females carrying broods of parthenogenetic eggs were commonly observed in the larger size classes (Fig. 4, Table 1). A few ephippial females were observed, but only during the later sampling dates (Figs. 3c, 4). Pond 8A contained large and small males throughout the sampling period (Fig. 5). Pond 8B was dominated by small males throughout most of the sampling period and larger males were observed in the later samples (Fig. 5). The proportion of mature males (> 0.76 mm) relative to ephippial females was always strongly biased towards males [8A: 0.82 ± 0.04 (mean ± SE); 8B: 0.90 ± 0.04]. An examination of 30

Table 1 Proportion of adult females (> 1.5 mm) carrying a brood, and mean brood size for *Daphnia pulex* from ponds 8A and 8B sampled in May 1992

Date		Pond 8A				Pond 8B			
		n	Proportion with a brood	*n*	Mean brood size (SE)	*n*	Proportion with a brood	*n*	Mean brood size (SE)
May	3	119	0.56	48	13.04 (0.99)	16	1.00	47	41.91 (1.59)
	6	73	0.66	24	24.33 (2.17)	4	1.00	25	46.16 (1.60)
	10	97	0.43	23	25.78 (1.36)	147	0.35	24	30.67 (3.17)
	14	4	0.75	34	28.29 (1.63)	90	0.48	36	22.94 (2.23)
	18	23	0.23	36	25.88 (0.94)	2	0.50	34	16.85 (0.99)
	21	61	0.28	–	–	52	0.19	–	–

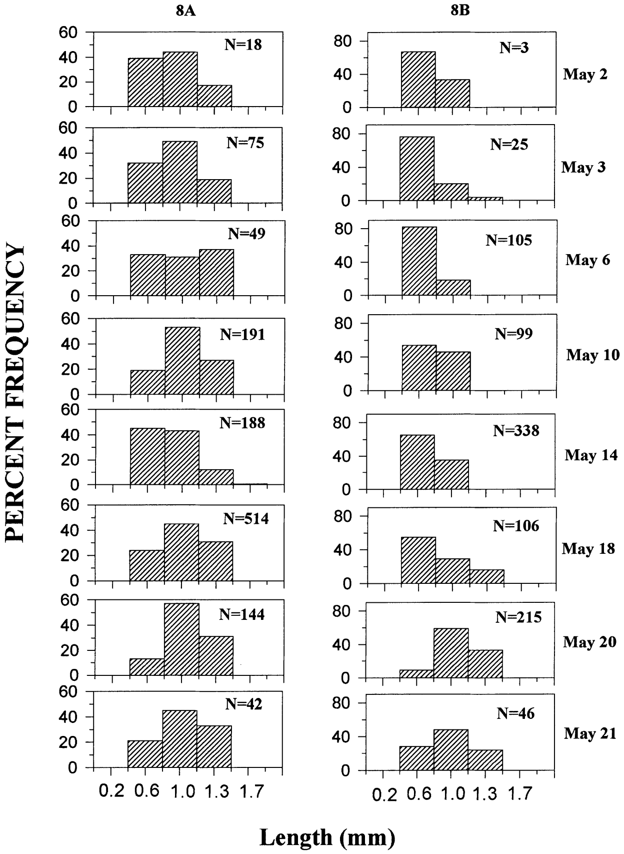

Fig. 5 Length frequency distributions for males sampled from ponds 8A and 8B sampled on eight dates in May 1992

ephippial females in pond 8A on May 6 showed that all were carrying two resting eggs.

Brood sex

Pond 8A showed an average proportion of male broods of 0.51 for the first three sampling dates but showed a significant $(G = 20.27, df = 4, P < 0.001)$ decrease in the proportion (mean = 0.17) of male broods for the last two samples (Fig. 3b). The proportion of male broods produced in pond 8B was relatively constant $(G = 3.54, df = 4, P > 0.05)$ over the sampling period (mean = 0.3; Fig. 3b). The total sample of all broods $(n = 333)$, collected on May 3, 6, 10, 14 and 18, consisted of 59% female, 30% male and 11% mixed sex. The average size of each type of brood (female = 26.91± 1.00; male = 26.85 ± 1.55; mixed = 28.32 ± 2.03) was not significantly different $(F_{2,329} = 0.17, P > 0.05)$.

Forty-seven females that had been isolated into test tubes to determine brood sex on May 14 released second and third broods. Most of the additional broods (F: female, M: male, X: mixed sex) were female with seven females releasing mixed-sex broods: 15 F-F, 1 F-M, 2 M-F, 3 M-M, 14 F-F-F, 2 M-M-M, 1 M-F-M, 2 M-F-F, 3 F-X, 1 X-F, 1 X-M-M, 1 X-F-F, 1 X-F-M.

Table 2 Frequency of non-male-producing females of *D. pulex* in two ponds sampled in 1991 and 1992. Sample sizes in parentheses

Year	Pond	
	8A	8B
1991	0.42 (111)	0.34 (95)
1992	0.53 (95)	0.44 (96)

Field cages

The interval between broods in the field cages averaged 5 days (range 4–8 days). Most of the information was limited to two or three successive broods due to mortality. A total of 58 of the 72 caged females survived and were assessed for the sex of successive broods from both ponds 8A and 8B. The number of females and the sex of the successive broods were as follows: 27 F-F, 4 F-M, 2 M-F, 7 M-M, 2 F-F-F, 1 M-M-M, 2 F-M-M, 4 X-M, 2 X-F, 2 F-X, 1 X-X, 1 M-X, 1 X-M-M, 1 F-X-M, 1 F-X-X.

Sex of laboratory-produced broods

Random samples of females collected from both ponds 8A and 8B on April 22, 1991 and May 18, 1992 were monitored for the frequency of NMP females under crowding in the laboratory (Table 2). There were no significant differences $(G = 8.14, df = 4, P > 0.05)$ in the frequency of NMP females between ponds and between years. In addition, 19 females that had produced two successive broods of females in the field cages in 1992 were also monitored for male production under crowding in the laboratory. The percentage of NMP females (89%) among the females that produced two successive broods of females in the field cages was significantly greater $(G = 13.2, df = 1, P < 0.001)$ than the percentage of NMP females (48%) from the random sample of females from both ponds.

Discussion

D. pulex populations in temporary pond habitats are characterized by a very early investment in sexual reproduction compared to *Daphnia* species in more permanent habitats, such as lakes (Mort and Wolf 1985, 1986; Hebert 1987; Jacobs 1990; Mort 1991; Spaak 1995). Males, and a high frequency of male broods, occurred very soon after the populations were reestablished from resting eggs in the early spring. Laboratory observations of the hatching of 700 resting eggs of *D. pulex* in a previous study (Innes and Dunbrack 1993) confirmed that only females hatch from resting eggs. Therefore, the early presence of males suggests that males were being produced during the first few broods of ex-ephippial (newly hatched) females. The appearence of males during the initial stage of population growth also indicates that density is probably not the only factor inducing the development of parthenogenetic eggs into males. Barker and Hebert (1986, 1990) also questioned the role of population density in sex expression in *D. magna*, since females exposed to high density in nature failed to exhibit a male bias among broods compared to females exposed to low density. Food limitation has also been implicated as a factor promoting the development of males (Banta 1939; Hebert 1978). However, the role of stress imposed by food limitation, for the production of males in *Daphnia* species, is unclear. Chemical factors, released during crowding, appear to be as or more important for inducing the development of males than food limitation in some experiments (Hobaek and Larsson 1990; Kleiven et al. 1992; Korpelainen 1992). Although phytoplankton abundance was not measured directly in ponds 8A and 8B, neither population showed any sign of stress from food limitation, as demonstrated by the high frequency of mature females carrying broods and the large clutch sizes. Previous observations on *D. pulex* populations confirm that these temporary-pond habitats are not food limited with respect to the intensity of parthenogenetic reproduction (Brambilla 1982; Stirling and McQueen 1986). Thus, the initial density and food conditions occurring in these ponds were not expected to stimulate male production, suggesting that the investment in male production is somewhat independent of environmental conditions.

Schwartz and Hebert (1987) found a similar early investment in sexual reproduction, as measured by the occurrence of males, in the cladoceran *Daphnia ephemeralis*. *D. ephemeralis* is often found coexisting in temporary ponds with *D. pulex* during the early spring. *D. ephemeralis* hatches prior to *D. pulex* and completes its sexual life cycle within several weeks. The two species co-exist for only a few weeks. *D. ephemeralis* populations exhibited sex ratios of approximately 19.4–76% males. The initial brood composition for *D. ephemeralis* averaged approximately 31% male, 51% female and 18% mixed-sex broods. Thus, both *D. ephemeralis* and *D. pulex* exhibit a very similar emphasis on sexual reproduction in these temporary-pond habitats. One major difference was the observation of males hatching from resting eggs in *D. ephemeralis*, which has not been documented in other species of *Daphnia*. No information on the occurrence of sexual females was reported, suggesting that males were the dominant sexual stage, at least initially. Observations by Stirling and McQueen (1986) also showed that the first few broods in the early spring consisted of males and females produced by overwintering females of *D. ephemeralis*. Males were observed prior to ephippial females.

In the current study, sexual females of *D. pulex* were commonly observed only after males had been produced. This was particularly striking in pond 8B where no sexual females were observed when the proportion of males was about 20%. Males were generally more common than sexual females in both ponds. The earlier production of males than sexual females and the high relative frequency of males compared to sexual females has also been observed for natural populations of *D. pulex* (Lynch 1983, 1984), *D. magna* (Korpelainen 1992) and *Daphnia galeata-cucullata-hyalina* complex (Spaak 1995). However, Young (1979) observed the occurrence of sexual females of *D. magna* when males were very rare. It would be interesting to know how many of the ephippial females had mated and were actually carrying resting eggs. In a small sample of ephippial females in the present study, all were carrying resting eggs, which is not surprising given the high frequency of males.

Yampolsky (1992) questioned the practice of expressing sex ratio as the ratio of males to the total population of females, including parthenogenetic (nonsexual) females as calculated by Barker and Hebert (1986) and Hobaek and Larsson (1990). Yampolsky (1992) suggested that the correct sex ratio is the ratio between males and sexual females. This ratio appears to be consistently biased towards males in ponds 8A and 8B, and in other populations of *D. pulex* (Lynch 1983, 1984) and *D. magna* (Korpelainen 1992). A male-biased sex ratio may, in part, be related to the unpredictable environment coupled with the influence of both genetic and environmental factors on sex expression. The occurrence of males prior to sexual females may be associated with the fact that parthenogenetic females can switch to sexual egg production very quickly, but newly released males require several molts before becoming sexually mature. Yampolsky (1992) observed that it would take only 3 days for a population to switch from 0% to 50% ephippial females compared to about 10 days for mature males to be produced. Early production of males would increase the probability of males maturing in time to participate in mating in an unpredictable environment. It is also possible that the presence of males may stimulate the production of sexual females as a mechanism for synchronizing the occurrence of both sexes. Both of these explanations require further observations in order to interpret the observed sex ratios within the context of sex allocation theory.

Lifetime allocation to male and female progeny for individual females is very difficult to establish under natural conditions. The limited data collected from the caged females in the present study suggested that individual females vary in sex allocation. However, because each female was not replicated it is not clear how much of the observed variation in sex allocation was simply due to random error and how much to genetic differences among females. Nevertheless, some females produced two and three successive broods of males, suggesting that these females have a higher investment in male progeny compared to females producing two and three successive broods of females. Follow-up experiments in the laboratory showed that females producing two successive broods of females in the field cages consisted of a higher frequency of NMP females than a random sample of females. It is therefore likely that some of the sex allocation variation measured under laboratory conditions can be applied to variation in the natural population. These results also suggest that sex allocation in *D. pulex* is not as simple as a regular alternation of male and female broods observed in *D. magna* (Barker and Hebert 1986; Hobaek and Larsson 1990; Kleiven et al. 1992). Sex allocation through the production of mixed-sex broods is also rarely observed in nature (Barker and Hebert 1986; Schwartz and Hebert 1987). However, in the present study, mixed-sex broods occurred with a frequency of about 11%. There is presently no information on the conditions that result in mixed-sex broods or on whether the occurrence of mixed-sexed broods has a genetic component.

Sexual reproduction in *Daphnia* has only recently been studied on the basis of sex allocation theory. The common occurrence of sexual reproduction in temporary habitats and the ease with which *Daphnia* can be cultured makes these species useful for studies on sex allocation variation in an organism with both genetic and environmental influences on sex expression. Brood size appears to be independent of offspring sex, which implies that male and female offspring are of equal resource content. Thus, variation in allocation to male and female function can easily be assessed by simply counting the number of offspring of each sex. Further studies using both laboratory and field observations are required to understand how particular patterns of sex allocation have evolved in these species. These studies should include information such as lifetime allocation to male and female progeny, and sexual eggs, for individual females throughout the seasonal existence of these temporary habitats.

Acknowledgements I wish to thank D. Singleton, J. Havel, S. Schwartz and L. Hermanutz for comments on the manuscript. L. Hermanutz assisted during the field sampling and D. Singleton, L. Fitzgerald and L. Mitchell assisted with counting the preserved samples. This research was supported by a grant from the Natural Sciences and Engineering Research Council of Canada and Memorial University of Newfoundland.

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