Inter-genotype variation in reproductive response to crowding among *Daphnia pulex*

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Abstract

Crowding is known to have a major influence on reproduction in the freshwater microcrustacean *Daphnia pulex*. We analyzed reproductive output of six different *D. pulex* genotypes under two different density regimes in the laboratory. Four of these genotypes reproduce via obligate parthenogenesis, allowing thorough analysis of the life history strategies of some asexual lines. Among 30,109 neonate offspring and 1041 resting egg ephippia collected, several trends were evident. Crowding induced increased resting egg production and reduced neonate offspring production among all genotypes. Offspring sex ratios grew more male-biased with maternal age. The extent, but not direction, of each of these trends varied among genotypes. Offspring sex ratios, and the very direction in which they changed in response to crowding, differed significantly among genotypes with some genotypes producing more and others fewer males in response to crowding. Obligately parthenogenetic genotypes seemed to respond to the crowding stimulus in similar ways as the facultatively parthenogenetic genotypes, as expected from the sexual origins of their genomes. The inter-genotype variation in life-history traits observed in this and other investigations calls into question the common practice of extrapolating results from a single *Daphnia* genotype to an entire species. Our findings are considered in the context of other research in the field of environmental influences on *Daphnia* reproduction with a review of representative literature.

Introduction

Reproduction does not necessarily follow the simple mantra of more is better. There are tradeoffs. Investing too much in reproduction early in life may leave an organism with fewer resources available for defence, growth, and future reproductive investment. The amount and type of reproductive output must also match the environment in which the offspring will grow. Thus the utilization of appropriate predictive cues can be just as important as reproductive investment itself.

The freshwater zooplankton *Daphnia pulex* (Leydig) (Crustacea: Cladocera) is well-suited to experimental research on life-history strategies. Like other model organisms, it is easily reared in the laboratory, has short generation times, and the basic components of its ecology and life-histories are known from previous research. It may reproduce via clonal free-swimming offspring (neonate) production or diapausing resting egg production (in ephippia), so trade-offs between short- and long-term reproductive investment can be observed. Clonal offspring production allows comparison of essentially the same genome across

several environmental conditions, which is a major advantage of using *D. pulex* in life-history research.

Crowding is known to affect reproductive type (neonate *vs.* resting egg) and amount among *Daphnia*. Crowding may precede one of several potential environments. Ephemeral populations of *Daphnia* tend to follow boom-and-bust cycles, whereby high densities often precede steep declines (Dudycha, 2004; Nelson et al., 2005). Crowding may also indicate decreasing water levels as a result of the pond drying up. Either of these situations should favour investment in diapause as opposed to neonate production. Accordingly, *Daphnia* have been found to produce more ephippia and fewer neonates in response to crowding (Berg et al., 2001; Lürling et al., 2003).

Crowding may also predict offspring environment to be more crowded and competitive, with potentially greater risk of horizontal transmission of disease and parasites. In this situation, whatever neonate offspring are produced should be of overall high quality, being resistant to food stress and parasites. Investment in higher-quality offspring may necessitate a concomitant decrease in offspring quantity. Research suggests crowded *Daphnia* mothers respond to crowding in similar ways as to food stress (Burns, 1995). Crowded mothers produce fewer neonates than uncrowded mothers, but these offspring tend to be larger (Gliwicz & Guisande, 1992; Cleuvers et al., 1997), develop more rapidly (Cleuvers et al., 1997), and be more resistant to starvation (Gliwicz & Guisande, 1992) and parasites (Mitchell & Read, 2005). Whether the production of high-quality neonates is a response to crowding in specific or to stresses in general remains to be determined. *Daphnia magna* Straus have been found to produce offspring with greater resistance to toxins, for instance, both when the mother is exposed to crowding (Baird et al., 1991) and to toxins (Gustafsson et al., 2005).

Several studies have examined the effect of crowding on various aspects of *D. pulex* reproduction (e.g., Ruvinsky et al., 1986; Innes et al., 2000; Berg et al., 2001). Our research explored the effects of crowding on *D. pulex* reproduction in terms of amount, mode (neonate *vs.* ephippia), and neonate sex ratio. Unlike some other research, we utilized six different genotypes (or clones,

henceforth used interchangeably) in order to compare genotypes' strategies and their responses to crowding (genotype×environment interactions). Genotype×environment interactions are known to have a great effect on *Daphnia* fitness and life-histories (Mitchell et al., 2005), as they likely do for most species. We also observed reproductive output in relation to maternal age, allowing age-specific strategies to be revealed.

Life cycle of *Daphnia pulex*

The life cycle of *D. pulex* must be considered in the context of its habitat; ephemeral freshwater ponds. It is only the resting egg stage of the *D. pulex* life cycle that can survive over winter or when the pond has dried up. *Daphnia* resting eggs may hatch the following year, or may remain buried and hatch many years later (Kerfoot & Weider, 2004). Normally two resting eggs are encased within an ephippium, which is a hard dark case that protects the eggs from stresses.

Daphnia pulex individuals can reproduce either by facultative parthenogenesis (FP) or obligate parthenogenesis (OP). Facultative parthenogenesis is also referred to as cyclical parthenogenesis in much of the literature, but a change in terminology to FP has been suggested (Tessier & Cáceres, 2004). Both FP and OP *D. pulex* readily produce neonates clonally, but FP lines require sex and recombination to produce resting eggs, while OP line females produce resting eggs asexually with resulting genotypes matching those that would be expected from mitosis (Hebert, 1987). Method of reproduction is believed to be inherited genetically (Innes & Hebert, 1988). Although many northern OP lines are polyploid (Hebert, 1987), allozyme electrophoresis results (not shown) led us to believe the genotypes used here were diploid.

Some OP lines are capable of producing males (Hebert et al., 1989; Innes et al., 2000). These males are functional, and can mate with FP females to produce a mixture of FP and OP offspring (Innes & Hebert, 1988). Although a clonal line may be obligately parthenogenetic, it will retain much of the genetic history of sexual *D. pulex* (Lynch et al., 1989). Cues to induce male production, for instance, may no longer be relevant to OP lines, but with genomes that are essentially

frozen OP lines may remain responsive to these cues.

Neonate sex is determined environmentally with genetic contributions (Innes & Singleton, 2000). Thus both male and female offspring are genetically identical. Neonates are produced in broods that range in size from several to dozens. All neonates within a brood are usually the same sex, although mixed-sex broods are not uncommon (Innes, 1997). Male and female neonates resemble small versions of their adult equivalents, and can be distinguished morphologically based on the presence of a distinct second antennule among males.

A key component of *D. pulex* fitness is a genotype's ability to produce an optimal number of offspring via neonate production to maximize the number of resting eggs produced before pond conditions deteriorate. The cues used by *D. pulex* to induce male- and resting-egg production vary across genotypes within and between habitats, and include crowding (Berg et al., 2001).

Materials and methods

Clonal isolates

Specimens were sampled from four ephemeral ponds in the Great Lakes watershed of North America in May 2003, using previously described methods (Innes, 1997). Disp and VBA ponds are in Windsor and Guelph, Ontario, respectively (Fitzsimmons & Innes, 2005). Morg and War are near Ann Arbor, Michigan, being previously described as ponds 69 and 73 respectively (Hebert & Crease, 1983). All ponds are similar in size and are believed to support *D. pulex* populations in the spring of each year only. Disp and Morg occur in woodlots, while VBA and War ponds are in more open areas. At least a dozen clonal isolates were individually maintained from each of these ponds in synthetic zooplankton media (Lynch et al., 1986) in conditions described elsewhere (Innes & Dunbrack, 1993). Cups of clonal strains were fed 3.5 mL of algal slurry from an aquarium system daily. The goldfish in the aquarium were fed fish food flakes, and their excretions provided nutrients for a small variety of phytoplankton and bacteria to thrive. Though the small random fluctuations in this algal community prevent precise quantifications, its multi-taxa composition is a more natural food than single algal strains, and may increase *Daphnia* reproductive output (Sanders et al., 1996).

The clones chosen for the experiment had been observed with males on multiple occasions in the lab. This non-random sampling of genotypes was purposeful, as only genotypes capable of producing males were desired. One clone from each of Disp and VBA was chosen, as were two clones from each of Morg and War. The two clones from Morg and War were genetically unique, as inferred from allozyme electrophoresis. It was originally believed that all six of these clones were OP, based on previous research with these populations (Hebert et al., 1989) and phenotypic heterozygosity at the lactate dehydrogenase (LDH) allozyme locus, which is usually correlated with obligate parthenogenesis (D. J. Innes, personal observation). Verification of mode of reproduction, however, strongly suggested the clones Disp and War2 were FP. Verification entailed allowing females to produce ephippia in the absence of males. OP daphnids can produce diapausing resting eggs within ephippia in the absence of males, whereas FP daphnids can only produce an empty ephippium (Innes et al., 1986). Since OP daphnids may also occasionally produce empty ephippia, however, a single empty ephippium is insufficient evidence for facultative parthenogenesis. More than 20 ephippia were opened for each of Disp and War2 without the presence of resting eggs, while ephippia containing resting eggs were found for each of the other clones within the first 3 being opened. We therefore assume Disp and War2 to be FP.

Additional FP and OP genotypes were included in the experiment initially, and would have allowed more general tests of FP vs. OP reproductive investment. However the amount of time required for accurate counting and sexing neonates necessitated a reduction in the number of genotypes tested. We do not feel it prudent to test for general differences between FP and OP *D. pulex* reproductive investment from such a small sample.

Three successive parthenogenetic generations of each clone, originating from a single female of each clonal culture, were raised in identical conditions in an incubator prior to the experiment. This was done to control for any potential

maternal or grandmaternal effects and to prevent mortality of clones unaccustomed to lab conditions (Antunes et al., 2003). These conditions were 15 °C, 16L:8D photoperiod, 3.5 mL of algae daily, and 5 females per 80 mL cup of media. Female neonates were obtained from the final generation within 24 h of being released from a brood and used in the experiment.

Experimental set-up

Translucent 140 mL plastic cups were filled with 40 mL of synthetic zooplankton media. Either 1 or 10 female neonates of a clone were included in a cup, for Alone and Crowded conditions, respectively. It should be noted that even the Alone condition may be considered somewhat crowded to *Daphnia* (Banta & Brown, 1929; Hobæk & Larsson, 1990). Eight replicate cups were used for each clone in each density condition (2 density conditions×6 clones×8 replicates = 96 cups). Cups were maintained in incubators at 15 °C, with a short-day photoperiod of 8L:16D. Alone and Crowded cups were fed 1.9 and 3.8 mL of algae daily respectively. Crowded cups were thus fed 2× as much algae, but had 10× more daphnids, and the results of our experiment may be either due to differences in density or food levels. We note that stock cultures of several dozen reproducing daphnids were maintained on only 2 mL of algae daily, suggesting food levels were ample in both treatment conditions.

Every other day the daphnids were transferred by pipette to a cup of new media, and the contents of the old media poured through a 250 μm plankton mesh to collect all neonates and ephippia. Neonates were temporarily immobilized in a shallow, weak ethanol-water solution to allow easier visualization and counting under a dissecting microscope. Neonate sex was determined based on visualization under the microscope. The numbers of male and female neonates were recorded along with number of ephippia. Ephippia were not opened to count the number of resting eggs within. After data collection, neonates and ephippia were discarded.

Data collection continued until the specimens reached the age of 38 days, at which point the experiment was terminated. Although *D. pulex* individuals can live longer than 1 month in a lab environment, their lifespan is likely shorter than 1 month in their natural habitat (Dudycha, 2004), so it made little sense to extend the experiment beyond 38 days of age. Spare replicates were kept for most clones, under identical conditions as others, to replace dead experimental daphnids. No daphnids in the Alone condition died in the experiment, but a small number died in the Crowded condition. Most of these were immediately replaced with individuals of the same genotype, age, condition, and stage of reproduction (i.e., carrying ephippia, carrying early-stage brood, etc.). This resulted in balanced statistical analyses.

Data analyses

All data were analyzed using MINITAB 14.1, and all graphs were produced using Prism 4.0. The General Linear Model (GLM) was the primary method used to evaluate each of the four major aspects of the data: Ephippia production, experiment-long fecundity, brood size, and sex ratio.

To test the various factors influencing variation in ephippia production, the factors of Density and Clone were included along with their interaction. The response variable was the total ephippia production over the length of the experiment per adult female in experimental cup. If ephippia production were no different between Alone and Crowded conditions, then per-female ephippia production would not differ between density conditions. Ephippia data were consolidated over the length of the experiment as opposed to analyzed at individual data points for two reasons: non-consolidated data produced residuals that violated all assumptions required for GLM, and Age was determined to not be a significant factor influencing ephippia production in these analyses (results not shown).

Total fecundity over the length of the experiment was calculated using neonate data only, since any incorporation of ephippia in fecundity would necessitate a weighting factor to be applied to it, which may or may not be accurate. With total neonate production per female over the length of the experiment as the response variable, terms in the model were Density, Clone and Density×Clone interaction.

The assumption is made in many *Daphnia* studies that male and female neonates cost the

same amount of resources to the mother (Korpelainen, 1992; Innes, 1997). This assumption is based largely on analyses by Barker & Hebert (1986) in which separate mixed model ANOVAs were performed on each of four clones of *D. magna*, testing for the influences of brood sex and date of brood release (i.e., age of mother) on brood size within each clone. In each of these analyses the date of brood release, but not brood sex, had a significant effect on brood size (Barker & Hebert, 1986). Since the sex of a brood had no significant effect on the number of neonates in the brood, males and females were assumed to cost the same resources for the mother. Similar within-clone comparisons of male and female brood sizes have been conducted on a single clone of *D. magna* (Hobæk & Larsson, 1990) and three clones of *D. pulex* (Innes & Singleton, 2000).

We tested our data in two ways, using a separate mixed model ANOVA for each relevant clone with the terms age of mother at brood release (Age, regression term) and brood sex (BrdSex) as was done by Barker & Hebert (1986), as well as using the GLM outlined below. Brood size could only be evaluated from daphnids in the Alone density condition, since separate broods could not be distinguished in the Crowded condition. The few mixed-sex broods observed in the Alone condition were excluded from analysis, so as not to confound the BrdSex factor. Because Morg2 and VBA did not produce both all-male and all-female broods in the Alone condition, all data for these clones were excluded from brood size analyses evaluating the effect of brood sex. We believe the GLM analysis evaluates the assumption of equal resource costs for male and female neonates in a more robust manner than clone-specific ANOVAs. The GLM incorporates all relevant clones' data into one model, and adds the Clone factor to determine the level of variation in brood size that can be explained by variation among clones. If our analyses were to find brood sex to significantly influence brood size, then assumptions of equal resource cost for male and female neonates would require re-evaluation.

The GLM to evaluate factors influencing brood size had the number of neonates in a brood (BrdSize) as its response variable. The terms in the model were: Age, Clone, BrdSex, and the two-way interactions Age×Clone, Age×BrdSex, and Clone×BrdSex. This GLM was evaluated using the same four clones mentioned above. If all factors involving BrdSex were found to be insignificant, then a new GLM would be created that would eliminate such terms and only include the factors Age, Clone, and their interaction. This GLM would include data from all six clones, since there would be no reason to exclude Morg2 and VBA for producing broods of one sex only.

Although sex ratio is a common subject of study, its analysis is difficult with *Daphnia*. Because all neonates within a *D. pulex* brood tend to be the same sex, assumptions of sex independence are violated for within-brood siblings. For this reason, a common way to analyze sex ratios or sex allocation in *Daphnia* research has been to classify each brood as either all-female, or containing any males (e.g., Hobæk & Larsson, 1990; Olmstead & LeBlanc, 2002). Because broods could not be distinguished from each other in the Crowded condition of our data, this method would not suffice for our purposes. Thus we totalled all neonate data across the length of the experiment for each replicate, producing lifetime sex ratios (total males over total neonates produced). This Sex Ratio was the response variable for our analysis, and the terms in the GLM were Density, Clone, and Density×Clone interaction.

Using consolidated data for sex ratios prevented the evaluation of Age as a factor influencing sex ratio variation. To evaluate its influence, another GLM was analyzed using only Crowded condition data, not consolidated over the length of the experiment. Data from the clone VBA were excluded since no males were produced in the Crowded condition. With Sex Ratio as the response variable, the terms of the model were Age (regression), Clone, and Age×Clone interaction.

To evaluate whether any effect of Age on sex ratios was due to an extraordinary bias of brood sex among first broods of *D. pulex*, we also compared sex ratio data between sequential broods. Since separate broods could not be distinguished in the Crowded condition, only data from the Alone condition were used. Data from Morg2 and VBA were excluded since neither produced both male and female broods in the Alone condition. Each replicate's broods were distinguished by the order in which they occurred. *t*-Tests of the brood data were carried out comparing the sex ratios of

first broods with those of second broods, and between second and third broods.

Results

A total of 30,109 neonates and 1041 ephippia were collected during the experiment (Table 1). Assumptions of residuals' independence, homogeneity, and sums of zero are made in any analyses based on General Linear Models (GLMs), and are met for all analyses here.

Ephippia production

Of the 1041 ephippia collected in this experiment, only 20 (1.92%) came from daphnids in the Alone condition, all of which were from the Morg2 clone (Table 1). This is reflected in the statistical results. The Density×Clone interaction explained a significant amount of the variation

in experiment-long ephippia production per adult female ($p < 0.001$, Table 2). The individual terms Density and Clone also explained significant amounts of variation ($p < 0.001$ for each, Table 2), but this must be interpreted with caution since the interaction term is significant and takes precedence. The extent of the increase in number of ephippia produced from Alone to Crowded conditions depended upon the clone (Fig. 1).

Fecundity

The total number of neonates produced by each daphnid varied according to both clone and density condition, with crowded individuals of each clone producing fewer neonates per-daphnid than those in the Alone condition (Table 1, Fig. 2). The interaction Density×Clone explained a significant amount of the variation in neonate production ($p < 0.001$, Table 2). The individual terms Density

Table 1. Numbers of neonates and ephippia collected in the experiment

Clone	Density	Male neonates	Female neonates	Total neonates	Sex ratio (% males)	Ephippia
Disp (FP)	Alone	453	114	567	79.89	0
	Crowded	2925	585	3510	83.33	42
	Total	3378	699	4077	82.86	42
Morg1 (OP)	Alone	71	1154	1225	5.80	0
	Crowded	730	2163	2893	25.23	179
	Total	801	3317	4118	19.45	179
Morg2 (OP)	Alone	10	463	473	2.11	20
	Crowded	42	332	374	11.23	345
	Total	52	795	847	6.14	365
VBA (OP)	Alone	2	1558	1560	0.13	0
	Crowded	0	6599	6599	0.00	95
	Total	2	8157	8159	0.02	95
War1 (OP)	Alone	553	1194	1747	31.65	0
	Crowded	3060	4897	7957	38.46	70
	Total	3613	6091	9704	37.23	70
War2 (FP)	Alone	1589	101	1690	94.02	0
	Crowded	919	595	1514	60.70	290
	Total	2508	696	3204	78.28	290
Overall	Alone	2678	4584	7262	36.88	20
	Crowded	7676	15,171	22,847	33.60	1021
	Total	10,354	19,755	30,109	34.39	1041

Clonal method of resting egg production is designated as facultative parthenogenetic (FP) or obligately parthenogenetic (OP). Daphnids were kept at densities of one (Alone) or ten (Crowded) in 40 mL of liquid.

Table 2. General Linear Model results for several analyses

	Factor	d.f.	Seq SS	Adj MS	F	p
Ephippia	Density	1	74.016	74.016	248.13	< 0.001
	Clone	5	119.306	23.861	79.99	< 0.001
	Density×Clone	5	27.302	5.460	18.31	< 0.001
	Error	84	25.056	0.298		
	Total	95	245.681			
Fecundity	Density	1	256739	256739	294.87	< 0.001
	Clone	5	187254	37451	43.01	< 0.001
	Density×Clone	5	65517	13103	15.05	< 0.001
	Error	84	73136	871		
	Total	95	582647			
Brood Size	Age	1	23718.4	19895.4	366.16	< 0.001
	Clone	5	14356.4	543.2	10.00	< 0.001
	Age×Clone	5	8354.6	1670.9	30.75	< 0.001
	Error	248	13475.3	54.3		
	Total	259	59904.7			
Sex Ratio	Density	1	0.03109	0.01089	0.64	0.427
	Clone	5	9.33902	1.87192	109.60	< 0.001
	Density×Clone	5	0.99187	0.19837	11.61	< 0.001
	Error	82	1.40054	0.01708		
	Total	93	11.76251			

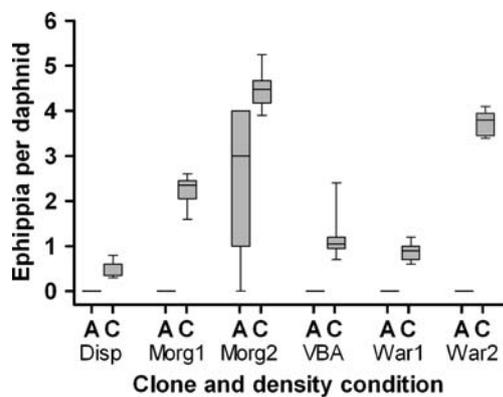See Methods (Data Analyses) for details on each analysis and factor.

Figure 1. Per-daphnid ephippia production among clones in the Alone (A) and Crowded (C) conditions. Boxes include the median and span the 25th–75th percentiles. Whiskers span the range of the data.
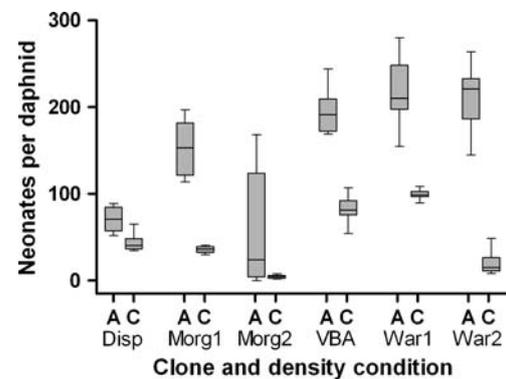

Figure 2. Experiment-long fecundity per daphnid among clones in the Alone (A) and Crowded (C) conditions. Boxes include the median and span the 25th–75th percentiles. Whiskers span the range of the data.

and Clone also explained significant amounts of this variation ($p < 0.001$ for each, Table 2). All GLM-based assumptions of residuals were met. As would be expected, there was a significant negative correlation among Crowded condition daphnids between the numbers of neonates and ephippia produced over the length of the experiment (Pearson correlation $r = -0.799$, $p < 0.001$, Fig. 3). A daphnid can carry either a brood of neonates or an ephippium at one time, resulting in a trade-off.
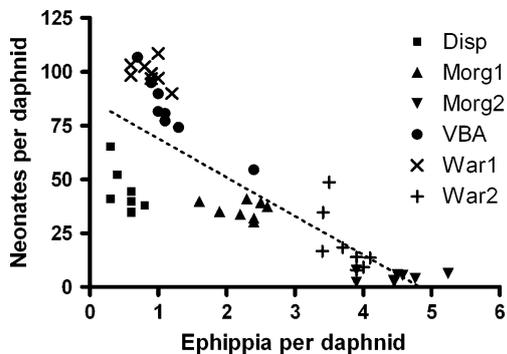
Figure 3. Experiment-long production of neonates and ephippia per daphnid among clones in the Crowded condition. The six different clones are designated by points of different shapes, with eight replicates each. The black dotted line represents the linear regression among all data points.

Brood size

Like Barker & Hebert (1986), we tested for the influence of brood sex and age on brood size in separate ANOVAs for each of the four appropriate Alone-condition clones in our data set. Age did ($p < 0.001$ for each clone, Table 3), and brood sex did not ($p > 0.05$ for each clone, Table 3), explain a significant amount of brood size variation in each of the four clones analyzed. This same pattern held

Table 3. Results of ANOVA analyses of brood size variation for each of four individual clones with respect to the factors of maternal age and brood sex

Clone	Factor	d.f.	Seq SS	Adj MS	F	p
Disp	Age	1	191.53	246.13	16.26	< 0.001
	BrdSex	1	54.74	54.74	3.62	0.064
	Error	42	635.73	15.14		
	Total	44	882.00			
Morg1	Age	1	3268.1	3168.5	47.20	< 0.001
	BrdSex	1	2.6	2.6	0.04	0.846
	Error	39	2618.3	67.1		
	Total	41	5889.0			
War1	Age	1	7663.8	7396.8	127.59	< 0.001
	BrdSex	1	79.7	79.7	1.37	0.248
	Error	39	2261.0	58.0		
	Total	41	10004.4			
War2	Age	1	4266.6	3566.2	80.27	< 0.001
	BrdSex	1	0.5	0.5	0.01	0.913
	Error	47	2088.2	44.4		
	Total	49	6355.4			

true when Age was substituted with the reproductive event order (i.e., each brood or ephippium is a reproductive event and ranked sequentially, data not shown). These results suggest the assumption of broods of each sex being the same size, and that there is no significant difference in resource costs for the mother between male and female neonate offspring, is valid.

The GLM evaluating effects on brood size among Alone condition data for the four appropriate clones failed to reveal significant effects of any term involving BrdSex. Thus, all terms involving BrdSex were discarded and a new GLM was evaluated incorporating all six clones. In this GLM, the interaction Age×Clone explained a significant amount of variation in the number of neonates in each brood ($p < 0.001$, Table 2). The terms Age and Clone alone also explained a significant amount of this variation ($p < 0.001$ for each, Table 2), but should be interpreted only after their interaction is considered. All GLM-based assumptions of residuals were met. The size of most clones' broods increased with age (Pearson correlation $r = 0.484$, $p < 0.001$), albeit to different degrees (Fig. 4). Individuals of the Disp clone, however, produced slightly smaller broods with age (Fig. 4a). Age×Clone remained significant even when Disp was removed from the analysis ($F_{4,202} = 6.98$, $p < 0.001$), as did the individual terms Age ($F_{1,202} = 357.78$, $p < 0.001$) and Clone ($F_{4,202} = 4.54$, $p = 0.002$).

Sex ratio

The sex ratio of all neonates produced over the length of the experiment varied among clones, and each clone responded differently to crowding. Some clones responded in opposite ways. Morg1 produced a greater proportion of males, while War2 reduced its proportion of males in the Crowded condition (Fig. 5). The interaction of Density×Clone was responsible for a significant amount of the variation in experiment-long sex ratios ($p < 0.001$, Table 2). The term Clone alone also had a significant influence on sex ratios, but as with other analyses the interaction term should take precedence over solitary terms if significant. All GLM-based assumptions of residuals were met.

To evaluate the potential influence of Age on sex ratios, non-consolidated Crowded condition
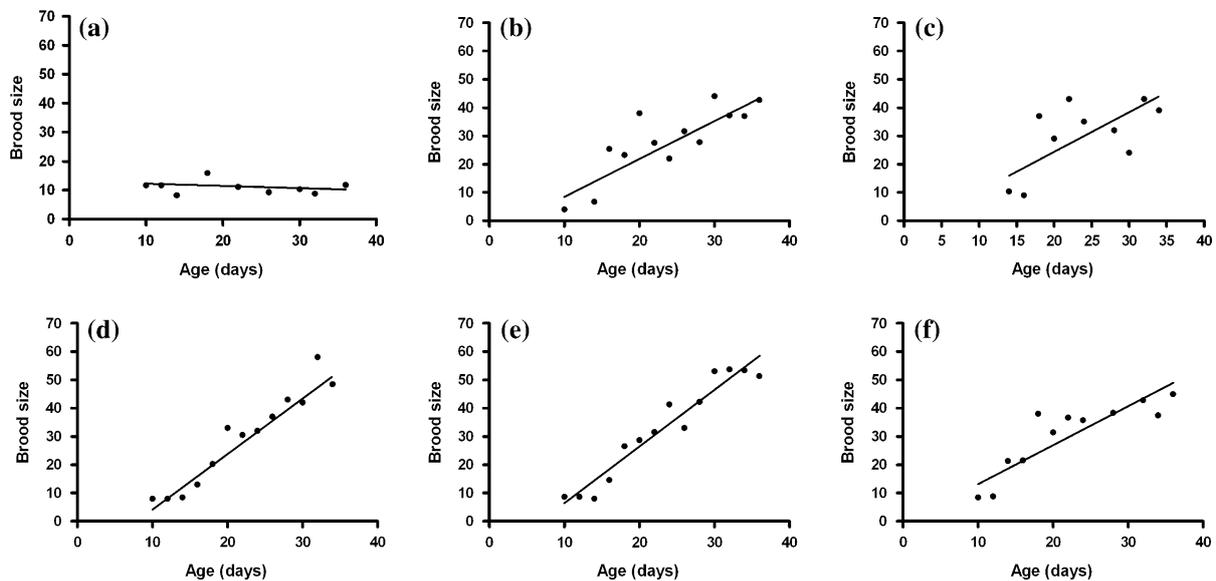
Figure 4. Number of neonates in broods of individuals in the Alone condition, plotted against age of the mother in days. Symbols plot the mean brood size among replicates at a given maternal age. Regression lines across maternal lifetime are shown. Graphs are separated by genotype: (a) Disp, (b) Morg1, (c) Morg2, (d) VBA, (e) War1, and (f) War2.
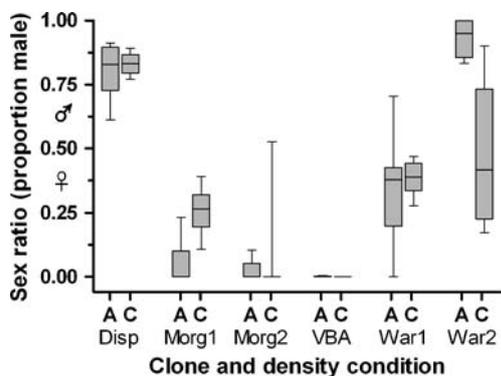

Figure 5. Overall sex ratios (proportion male) of all neonates among clones in the Alone (A) and Crowded (C) conditions. Boxes include the median and span the 25th–75th percentiles. Whiskers span the range of the data.

data were analyzed. Unlike neonate data from the Alone condition, which tended to be all-male or all-female, Crowded condition data were often intermediate due to several daphnids producing broods in the same time frame, often of different sexes. The Age×Clone interaction explained a significant amount of the variation in sex ratios according to our analyses ($F_{4,286} = 2.52$, $p = 0.041$, Fig. 6). Both Age ($F_{1,286} = 108.92$, $p < 0.001$) and Clone ($F_{4,286} = 7.99$, $p < 0.001$) alone also

explained significant variation in sex ratio. All GLM-based assumptions of residuals were met. Each of the five appropriate clones analyzed under Crowded conditions increased their sex ratio in favour of males with age (overall Pearson correlation $r = 0.391$, $p < 0.001$).

To further evaluate the influence of age on sex ratios, we considered whether the age effect was simply due to heavily female-biased first broods. At least one previous study had found no first broods to be male among the three *Daphnia* clones analyzed (Stross, 1969). Using brood data from the Alone condition we compared sequential broods' sex ratios using *t*-tests. Alpha levels were reduced to $\alpha = 0.025$ to reduce risk of type one error with two *t*-tests, as per the Bonferroni correction. We excluded from our analysis all mixed-sex broods and all data from the clones Morg2 and VBA for reasons explained above. Brood data from the remaining four clones were consolidated according to reproductive event, with a replicate's first brood or ephippium given a rank of one, its second reproductive event a two, etc. The mean and standard error sex ratios of broods as the first three reproductive events were 0.194 ± 0.072, 0.484 ± 0.091 and 0.600 ± 0.091, respectively. Broods that were daphnids' first reproductive event
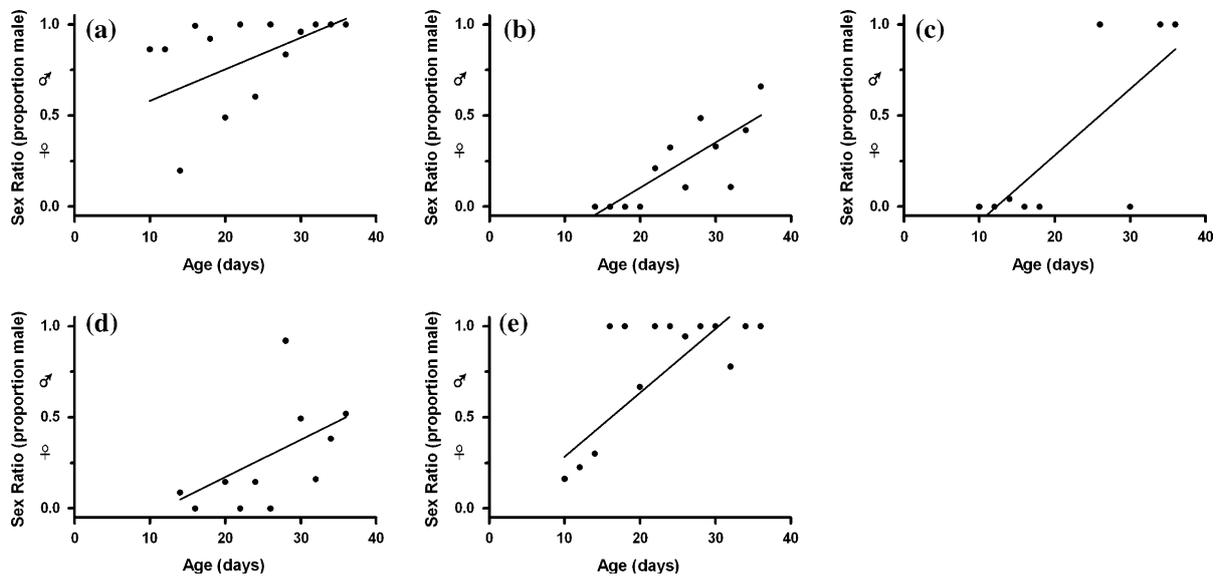
Figure 6. Sex ratio (proportion male) of neonate offspring of five clones in the Crowded condition, in relation to age of mother in days. Symbols plot the mean sex ratios among replicates at a given maternal age. Regression lines across maternal lifetime are shown. Graphs are separated by genotype: (a) Disp, (b) Morg1, (c) Morg2, (d) War1, and (e) War2.

were all-male on several occasions, but were significantly more female-biased than broods that were daphnids' second reproductive event ($t_{56} = -2.50$, $p = 0.016$). Each subsequent brood increased its propensity toward male production, but with insignificant differences between broods (sex ratio differences between second and third broods as reproductive events, $t_{58} = -0.90$, $p = 0.371$).

Abnormal neonate and mixed-sex brood analyses

A total of 114 of the 19,755 female neonates collected during this experiment (0.58%) were deformed morphologically as described in the Discussion. Only three of these abnormal neonates (2.63%) were observed in the Alone condition. Abnormal neonates were produced by each of the experiment's six clones. The assumptions based on residuals necessary for GLMs were violated, preventing its use for analysis of the factors influencing the proportion of female neonates that were deformed. Instead, correlations were analyzed with respect to the fraction of female neonates that were abnormal and the density of adult daphnids. There was a significant positive correlation between the density of adult daphnids and the fraction of female neonates that were abnormal (Pearson correlation $r = 0.108$, $p = 0.019$). Thus as

adult density increased, so did the proportion of daughters with developmental abnormalities. Age was significantly and negatively correlated with the fraction of female neonates that were abnormal ($r = -0.115$, $p = 0.012$), suggesting older daphnids produced relatively fewer abnormal daughters.

Among daphnids in the Alone condition in this experiment, 13 out of 278 broods (4.68%) contained both males and females. Other studies have found mixed-sex broods to occur at frequencies of ~11% for *D. pulex* (Innes, 1997), and 7.6% (Barker & Hebert, 1986) and 5.6% for *D. magna* (Kleiven et al., 1992) under various conditions.

There has been speculation as to whether or not there is a genetic component to the propensity to produce mixed-sex broods (Innes, 1997). With only 13 mixed-sex broods, the present data set is too small for formal analysis of influential factors. We simply point out that each of the six clones produced at least one mixed-sex brood, with War1 producing the most such broods with six (data not shown).

Discussion

Crowding provided an important stimulus in reproductive decisions of *D. pulex*. Both the type

and amount of reproduction varied across density conditions, but remained consistent within each condition within each genotype. This is yet another example of intraclonal phenotypic plasticity in response to environmental stimuli (Lushai et al., 2003; Printes & Callaghan, 2003). Different genotypes responded to the crowding stimulus in ways that were often similar but sometimes contradictory.

Reproductive responses to crowding

The per-daphnid rate of ephippia production was significantly higher for crowded daphnids than for those that were alone. In fact, only one of the experiment's six genotypes produced any ephippia in the Alone condition. Genotypes differed in the extent of their ephippia production increase with crowding, but all increased.

Other research has also found ephippia production among *D. pulex* to vary across clones (Innes et al., 2000; Tessier & Cáceres, 2004) and to increase as a result of crowding (Lürling et al., 2003), sometimes in a clone-specific manner (Berg et al., 2001). Research using other species of *Daphnia* has yielded similar results for inter-clonal variation in ephippia production among *Daphnia pulicaria* Forbes (Cáceres & Tessier, 2004; Tessier & Cáceres, 2004), and an increase in ephippia production with crowding among clones of *D. magna* (Ferrari & Hebert, 1982; Carvalho & Hughes, 1983), also in a clone-specific manner (Yampolsky, 1992). Some studies have found crowding to only increase ephippia production when combined with additional stimuli, such as reduced food levels (Olmstead & LeBlanc, 2001) or reduced food levels and low-light photoperiods (Kleiven et al., 1992), but these studies considered only a single clone so their broad applicability may not be valid (see below). Stimuli other than crowding have also been suggested to induce *Daphnia* ephippia production including: photoperiod (Deng, 1996, 1997b), food level (Deng, 1996), predatory fish (Ślusarczyk, 1995), density of males (Innes, 1997, Innes & Singleton, 2000), clonal competitive ability (Loaring & Hebert, 1981), parasite resistance (Mitchell et al., 2004) and the contrast between mother and offspring food levels (LaMontagne & McCauley, 2001). More multi-environment, multi-genotype research is required to evaluate the effects of interacting stimuli and genotype×environment interactions on ephippia production.

Just as the effects of crowding on ephippia production were uni-directional but differed in magnitude among clones, crowding reduced per-daphnid experiment-long fecundity in all clones, but to various degrees. This result is supported by previous research on *D. pulex* in which the total number of neonates produced per daphnid was reduced in response to Crowded conditions, but in a clone-specific manner (i.e., significant clone x density interaction) (Innes & Singleton, 1994; Innes & Singleton, 2000; Berg et al., 2001). Microparasites (Lass & Bittner, 2002), predatory fish (Weber & Declerck, 1997, Lass & Bittner, 2002), and temperature (Loaring & Hebert, 1981) are among the other factors that have been suggested to influence *Daphnia* fecundity with effects that often vary among clones.

As with other studies, we found a significant negative correlation between the total numbers of ephippia and neonates produced among daphnids in the Crowded condition because a daphnid may only carry an ephippium or a brood at one time (Loaring & Hebert, 1981; Ruvinsky et al., 1986; Yampolsky, 1992; Berg et al., 2001). This represents an intrinsic trade-off between neonate and resting egg production.

A fraction (0.58%) of the female neonates produced in this study were abnormal morphologically. They were characterized by their small size and deformed carapaces and antennae. These developmental abnormalities closely resemble those observed among neonates of *D. magna* individuals exposed to fenarimol (Mu & LeBlanc, 2002). Mu & LeBlanc (2002) further investigated the basis for the developmental abnormalities they observed, and found fenarimol exposure during embryo development to result in reduced ecdysone levels among neonates. We undertook no such biochemical tests of ecdysone levels among daphnids in our experiment. Age was negatively and adult density was positively correlated with the fraction of daughters that were deformed. Further research is required to thoroughly evaluate the embryological reasons for this developmental abnormality and the metabolic and ecological influences on its underlying factors.

The effect of crowding on brood sizes could not be determined in our experiment due to its design.

Other research has found *Daphnia* brood sizes to maximize at an intermediate level of crowding (Burns, 1995) and decrease with further crowding (Burns, 1995; Cleuvers et al., 1997; Boersma et al., 1999). Smaller brood sizes in response to crowding may be a result of the smaller size of mothers raised under Crowded conditions (Boersma et al., 1999), or the result of a trade-off between brood size and neonate quality (Mitchell & Read, 2005). We found brood size, like fecundity, to vary significantly among clones in the Alone condition. As most daphnids grew older, they produced broods with greater numbers of neonates. The magnitude of this age (and size) effect varied across clones (i.e., clone×age effect), with Disp daphnids notably not increasing their brood sizes with age. Previous research using two clones of *D. magna* found the linear increase of brood size with age to level off in old age (Glazier, 1992). Other research has found that the effect of age upon brood size is one that can be modified by other cues. Upon exposure to a microsporidian parasite, *D. magna* have been found to increase reproduction at young ages and decrease reproduction when old (Chadwick & Little, 2005). *Daphnia* brood size has also been observed to vary in response to food level (Gliwicz & Guisande, 1992) and exposure to cyanobacterial toxins (Gustafsson et al., 2005).

Offspring sex ratios

Because offspring sex is determined by non-genetic factors post-fertilization, *Daphnia* are classified as having environmental sex determination (ESD). Genotypic sex determination (GSD), in contrast, occurs at conception based on genes that are often carried on sex chromosomes (Valenzuela et al., 2003). Although some argue for continued and clear distinctions to be made between ESD and GSD systems (Valenzuela et al., 2003), there is growing support for viewing ESD and GSD as ends of a spectrum between which a continuous range of systems may lie (Sarre et al., 2004). Sex expression of *Daphnia* has been described as a result of genotype-by-environment interactions (Yampolsky, 1992; Innes, 1997; Innes & Singleton, 2000), a description our results support.

Fisher's rationale behind selection for a 1:1 operational sex ratio holds true only if sons and daughters cost equal resources to their mothers (Fisher, 1930; Carvalho et al., 1998). Therefore, the relative costs of male and female neonates to mothers must be considered before *D. pulex* sex ratios can be evaluated in full. Three previous studies have found no difference in sizes of male and female broods among clones of *D. magna* (Barker & Hebert, 1986; Hobæk & Larsson, 1990) and *D. pulex* (Innes & Singleton, 2000). One of these studies used only a single clone, so its results should be treated with caution (Hobæk & Larsson, 1990). The statistical methods used in the other two studies lacked the power of the one used here because they evaluated each clone separately.

Whether we used within-clone tests or all-clone models, we found no significant difference in the sizes of male and female broods among male-producing clones in the Alone condition. We therefore conclude that if there are differential costs in male and female neonate production, they are either minute or resources are redistributed from other functions to accommodate the more expensive sex. It is likely that male and female neonates cost the same to a mother, thus sex allocation and sex ratio of neonates can be considered equal.

One clone (VBA) was reluctant to produce males under either density condition, producing only 2 males among 8159 neonates. This was surprising, as numerous males had been observed in stock cultures of this clone prior to this experiment. This provides further evidence that the cues to male production vary across clones.

Among all five male-producing clones in Crowded conditions, there was a significant increase in the sex ratio in favour of males as daphnids grew older. This was true to such an extent that the two FP clones (Disp and War2) were producing male broods almost exclusively by the end of the experiment. Although the effects of seasonal progression on the ratio of males in ponds has been studied (Barker & Hebert, 1986; Innes, 1997), little research has explicitly examined changes in offspring sex ratio within the lifetime of individual daphnids. Increased male production with age has been observed with a single clone of *D. magna* (Hobæk & Larsson, 1990), and another study found no first-broods of three *Daphnia* clones to be male (Stross, 1969). The single *D. magna* clone used in a study by Olmstead & Le-Blanc (2001) only increased its male production

with age when under certain environmental conditions and the juvenile hormone analog methoprene was added; otherwise this clone reduced its male production with age.

More research is required into the effects of age upon offspring sex ratios, but we will speculate on potential selective pressures for increased male production with age. These pressures assume sexuality and focus on sexual selection. Sexual selection is of little current relevance to OP lines, but may still be entrenched among OP genomes due to their sexual origins.

We assume males to be more variable in reproductive output than females (Trivers & Willard, 1973). Little is currently known about sexual selection among *Daphnia* (Brewer, 1998) other than males can fertilize >20 females in one day, and mate with females regardless of genetic similarity (Winsor & Innes, 2002). Future research on pre- and post-copulatory sexual selection under a variety of conditions would be beneficial to our understanding of *Daphnia*. Below are three potential evolutionary reasons for increased male production with maternal age.

First, increased male-production with age may be a bet-hedging strategy, whereby males are only produced after it has been ensured that some females have been produced, since males are a riskier investment. It has been demonstrated mathematically that generations of low fitness affect the overall geometric mean of fitness more than those of high fitness (Vrijenhoek, 1998). Thus a 'safe' female-first strategy may be beneficial even if the various low- and high-reproductive generations of an early male production strategy would otherwise seem to cancel each-other out.

Second, assuming males are better able than females to translate high fitness into increased offspring production, if offspring quality increases with maternal age then selection should favour an increased male-bias with maternal age (Trivers & Willard, 1973). Offspring quality could increase with maternal age simply as a result of older, larger females being able to invest more resources into offspring. Indeed, a study using two genotypes of *D. magna* found females to produce eggs of greater mass with age (Glazier, 1992). In support of this resource-investment-in-males theory, bird offspring sex ratios increase in male-bias with increased maternal condition and/or maternal

food intake (Whittingham et al., 2005 and refs therein).

Finally, in addition to being able to allocate increased resources to offspring to increase their quality, an older mother may also indirectly ensure genetic quality for her offspring. A long-lived daphnid is generally of higher quality than those that died at a younger age. Therefore, increased male production among older daphnids may have been selected as a way for high-quality genotypes to produce the offspring sex that can best convert high genetic quality into increased reproductive output.

Offspring sex ratios of *Daphnia* are often reported to become male-biased when the mother is exposed to either Crowded conditions, or the cues within *Daphnia*-crowded water (Hobæk & Larsson, 1990; Kleiven et al., 1992; Innes et al., 2000; Innes & Singleton, 2000). It is often suggested that increased male production is induced by similar stimuli as ephippia production, just at a more relaxed level (Ferrari & Hebert, 1982, Kleiven et al., 1992). This is supported by *Daphnia* field sample studies finding high levels of males in a pond or lake 2 weeks before high levels of ephippial females (De Meester & Vanoverbeke, 1999; Cáceres & Tessier, 2004; Spaak et al., 2004). In addition to crowding, stimuli reported to alter *Daphnia* offspring sex ratios include photoperiod (Ferrari & Hebert, 1982, Korpelainen, 1986, Innes et al., 2000, Zhang & Baer, 2000), temperature (Korpelainen, 1986), food level (Ferrari & Hebert, 1982; Zhang & Baer, 2000), and exposure to predatory fish (Boersma et al., 1998).

In contrast to some of the literature cited above, our results do not support a simple relationship between increased crowding and increased male-bias among *D. pulex* sex ratios. We found significant differences not only among clones' sex ratios, but also among clones' sex ratio responses to increased density. The clones Morg1 and War1 increased their sex ratio in favour of males under Crowded conditions, while War2 behaved in the opposite way by producing fewer males under Crowded conditions. Disp, meanwhile, did not alter its sex ratio much either way with crowding.

How can our findings of inter-clonal variation in sex ratio responses to density be reconciled with the above-cited literature describing a simple

positive relationship between crowding and sex ratios? The experimental design of some studies prevented detailed quantification of offspring sex ratios in response to crowding (Innes & Singleton, 2000), or only studied the effects of crowding when combined with photoperiod and thus could not tease apart the effects of the two cues (Innes et al., 2000). Two studies used the same single genotype of *D. magna* and thus could not detect inter-clonal variation (Hobæk & Larsson, 1990; Kleiven et al., 1992).

Studies that have tested for inter-clonal variation in sex ratios have found it, among clones of *D. pulex* (Innes & Dunbrack, 1993; Innes, 1997; Innes et al., 2000; Innes & Singleton, 2000), clones of *D. pulicaria* (Tessier & Cáceres, 2004), and both clones (Korpelainen, 1986) and populations (Ferrari & Hebert, 1982) of *D. magna*. In fact, the significant clone×density interaction affecting sex ratios in the present study was also found to significantly influence sex ratios in other studies on *D. pulex* (Ruvinsky et al., 1986; Innes & Singleton, 1994) and *D. magna* (Yampolsky, 1992). A population×density interaction similarly influenced sex ratios in a component of a study on *D. pulex* that did not distinguish between clones (Berg et al., 2001). We are also not alone in reporting crowding to induce different clones to respond with sex ratios biased in opposite directions (Innes & Singleton, 1994).

Applicability of results from the laboratory to nature is difficult to assess. However, other researchers have found their lab results to match well with field results for *D. pulex* (Innes, 1997; Tessier & Cáceres, 2004), providing reason to believe the same may be true for the present experiment.

Male production among asexual D. pulex

Previous research suggests male production among OP genotypes is much-reduced compared to male production among FP genotypes (Innes et al., 2000). Any male production at all among OP clones seems paradoxical, but may in fact be an adaptive trait. Males from OP clones can mate with FP females to create novel OP lines (Innes & Hebert, 1988). There is evidence that obligate parthenogenesis has been spreading across North American *D. pulex* populations in a contagious fashion through such FP-OP matings (Paland et al., 2005). These new OP lines are expected to benefit from the half-genome contribution of the FP mother, as it is expected to be both locally adapted and less mutated than that of the OP father (Simon et al., 2003). Novel asexual lineages are believed to have resulted from similar matings between asexual-lineage males and sexual-lineage females in other species (Weinzierl et al., 1999, Simon et al., 2002).

Although selection may favour male production among OP clones in areas near FP populations of *D. pulex* (Innes et al., 2000), why would male production continue among OP clones far away from FP populations? Asexual genomes by their very definition do not recombine with others, and thus change very little over generations. Selection at each locus is reduced in an asexual genome (Rice & Chippindale, 2001; Betancourt & Presgraves, 2002; Hadany & Feldmans, 2005), so traits may be retained after they have ceased to be adaptive. Put simply, an asexual genome has trouble separating itself from its maker. This applies to sexual selection strategies (see above), male production, and other traits adaptive to sexuality.

It would be interesting to contrast levels of male production from OP populations at the FP-OP overlap range of middle North America with those from the Northeast of the continent whence obligate parthenogenesis is believed to have originated, and where obligate parthenogenesis is now ubiquitous among *D. pulex* populations (Paland et al., 2005). Is selection against male production in the Northeast strong enough to have eliminated those genotypes with heavy male production and hence reduce genotypic diversity? Or, is it weak enough that its effects have been largely overridden by the overall fitness of the rest of the genome, and from the benefits individual genotypes may enjoy in populations with low genetic diversity? Has sufficient time passed for selection against male production to have had an effect? It is also possible that males serve a yet-unknown selected function, and their continued production is in fact adaptive.

Daphnia pulex crowding infochemicals

Having discussed the effects of crowding on *D. pulex*, let us now consider the very nature of

this crowding stimulus. What are the infochemicals used by *Daphnia* to detect crowding, and how specific are they? Although physical interaction with other daphnids may increase the stimulus, the mere water in which daphnids have lived is sufficient to induce a crowding response among *Daphnia* (Larsson, 1991). Six kairomones have recently been isolated from *D. pulex* that induce morphological changes in algal prey (Yasumoto et al., 2005). Whether these kairomones are the crowding infochemicals detected by fellow daphnids remains to be seen, but their synthesis allows future research to test the possibility.

The specificity of *Daphnia* infochemicals is a matter of evolutionary importance. If daphnids were able to distinguish between infochemicals emitted by males and females then they could conceivably adjust their offspring sex ratios or reproductive behaviour accordingly, as some monoecious plants do depending on the amount of pollen they detect (López & Domínguez, 2003). The population demographics experienced by a mother, however, may not reliably predict those her offspring will experience at sexual maturity, which may be a reason why some bird species, for instance, do not employ this strategy (Bensch et al., 1999). A study using a single genotype of *D. magna* failed to reveal any distinction between male and female cues in swarming behaviour (Crease & Hebert, 1983).

If genotypes of *Daphnia* were able to distinguish between their own infochemicals and those of others in the same species, then they would be better able to determine outbreeding opportunities. Within-clone matings are known to produce offspring with fitness that is variable but reduced on average (Innes, 1989; Innes & Dunbrack, 1993; Deng, 1997a; Salathé & Ebert, 2003). Perhaps *D. pulex* adjusts its reproduction in response to outbreeding opportunities, just as female ambrosia beetles adjust their offspring sex ratio depending on outbreeding opportunities (Peer & Taborsky, 2004).

Are infochemicals detected across species within the genus *Daphnia*, or even within the family Cladocera? Inter-species detection of infochemicals decreases their reliability of prediction, and may select for the utilization of alternative predictors of environmental conditions (Serra et al., 2005). As with *Daphnia*, crowding induces a sexual response in many rotifers (Carmona et al., 1993). However, both experimental and theoretical evidence suggests some rotifers use the progression of generations as a sex-inducing cue instead of crowding, likely because crowding cues are not species-specific and hence lack accuracy (Serra et al., 2005). What little research conducted on inter-species detection of *Daphnia* infochemicals has used single genotypes, so inter-species response levels could not be compared to intraspecific inter-genotype response levels (Hobæk & Larsson, 1990; Burns, 1995; Lürling et al., 2003).

Many aspects of *Daphnia* infochemicals have implications that extend to experimental design. Much research has been conducted on the effects of fish-inhabited water on *Daphnia*, with effects attributed to fish kairomones (e.g., Boersma et al., 1998; Weetman & Atkinson, 2002). However, fish were almost always fed *Daphnia*, and evidence suggests it is not fish kairomones but latent daphnia alarm cues activated by bacteria and/or enzymes in fish digestive tracts to which daphnids respond (Stabell et al., 2003). A similar mechanism may be responsible for observed daphnid responses to water exposed to invertebrate predators (e.g., Pijanowska & Kowalczewski, 1997). And in a study on the effects of crowding upon *D. pulex* reproduction, water used for all clones came from one pond (Berg et al., 2001): a not-uncommon method among *Daphnia* laboratories (e.g., Deng, 1997b). The authors point out that this may have differentially affected the clone originating from this pond compared to the other clones in the experiment, but rate this an unlikely possibility (Berg et al., 2001).

Research implications and directions

This experiment and others like it have provided ample evidence for different genotypes of *Daphnia* responding differently to stimuli. A great deal of published research ignores inter-genotype variation, drawing species-wide conclusions from a single genotype of *Daphnia* (e.g., Kleiven et al., 1992; Alekseev & Lampert, 2001; LaMontagne & McCauley, 2001; Kessler & Lampert, 2004; Mikulski et al., 2005). Drawing species-wide conclusions from multiple replicates of the same genotype can be considered a form of pseudoreplication, and should be avoided (Hurlbert, 1984).

Testing regulations of freshwater effluent established by governments worldwide require no more than one genotype of *Daphnia* to be used (OECD, 1998; Environment Canada, 2000; USEPA, 2002). In fact, some researchers and regulations have advocated the widespread use of a single specific genotype to aid repeatability between laboratories (Baird et al., 1991; OECD, 1998).

By allowing tests to be conducted on a single genotype, government regulations are making an improper assumption of low inter-genotype variation. If repeatability is the justification for these regulations, then universal usage of a specific *battery* of genotypes would improve upon current regulations' repeatability. If labour costs to company laboratories for using several instead of single genotypes is the rationale behind current regulations, then we must re-examine the value we place on the well-being of our aquatic environment (De Meester & Declerck, 2005).

Conclusions

Crowding is an important stimulus to reproductive decisions of *Daphnia pulex*. Six genotypes of *D. pulex* were raised under two different density regimes and reproductive output was observed. Four of these genotypes reproduce via obligate parthenogenesis and two by facultative parthenogenesis. Insufficient sample sizes prevented prudent comparison of reproductive output between the two reproductive types.

Crowding induced increased ephippia production and decreased neonate production among all genotypes. Neonate brood sizes increased with maternal age for most genotypes, and offspring sex ratios grew more male-biased with maternal age for all relevant genotypes. Most daphnids modified offspring sex ratios in response to crowding, but in genotype-specific directions that were often contradictory. The genotype×environment interaction for sex ratios was the most obvious of several in this investigation. Inter-genotypic variation in response to environmental stimuli highlight the importance of using more than one genotype in *Daphnia* research.

Several potential evolutionary explanations are explored for increased male production with age, male production among asexual *Daphnia* and a potential environment-induced trade-off between offspring quantity and quality. We also discuss the nature of *Daphnia* crowding infochemicals, and comment on abnormal neonate development.

The crowding stimulus is an important one in *D. pulex* reproductive decisions, but one to which different genotypes may respond in different ways.

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