Effects of physical interference on life history shifts in *Daphnia pulex*

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SUMMARY

*Daphnia pulex* were reared in 50 ml flasks, each containing 1, 20 or 40 individuals, which were serially connected with a 20-μm mesh screens between, in order to examine the effect of physical interference due to crowding on shifts of life history traits throughout two consecutive generations. A flow-through system, designed to maintain a sufficient food supply and minimize the accumulation of metabolites, was used. To eliminate the effect of infochemicals from crowded animals, a single-individual treatment flask was connected to two crowded flasks. In the first generation, *D. pulex* reared under crowded conditions grew more slowly after day 4 when oogenesis normally starts, and produced less offspring after day 9, compared with an animal reared alone, even when supplied with sufficient food. Although second generation daphniids of each treatment matured faster than in the first generation, crowded females grew more slowly even after day 2 and produced less offspring than single females. Age to maturity was no different between treatments in both generations. Crowded females, therefore, matured to smaller sizes but produced larger neonates compared with single females. Weight-specific reproduction rates of the first clutch were not significantly different between the treatments. These results suggest that physical interference between neighboring individuals due to crowding negatively affects growth and reproduction in daphniids. Crowded daphniids may allocate more energy to reproduction in order to produce larger and more starvation-tolerant offspring in preparation for severe food shortages. Crowding also triggered ephippial egg production and reduced survival compared with the single-individual treatment.

Key words: crowding, growth, reproduction, *Daphnia pulex*, physical interference.

INTRODUCTION

Per capita growth is well known to decrease to some degree as population density increases under exploitative competition (Kerfoot et al., 1985; Begon et al., 1990). This phenomenon is thought to be due to resource limitation. Recently, such a response to high population density or crowding has also been explained by interference competition through allelopathic interaction, even under sufficient food conditions in cladocerans (e.g. Seitz, 1984; Matveev, 1993; Burns, 1995). For instance, Matveev (Matveev, 1993) found that water preconditioned with crowded *Daphnia carinata* for 30 h had the effect of reducing the feeding rates of *Daphnia carinata* and *Daphnia lumholzi*, and Burns (Burns, 1995) showed that water conditioned with crowded *Daphnia hyalina* and *Daphnia galeata* for 24 h adversely affected their growth and reproduction, even in the presence of excess food. Although the evidence for such chemically mediated effects on the demographic parameters in cladocerans has accumulated during the last decade (Gosser and Ratte, 1994; Mitchell and Carvalho, 2002; Lürling et al., 2003), conflicting evidence also exists. Water preconditioned with crowded conspecifics enhances the reproduction of *Daphnia hyalina* and *Daphnia cuculata* (Seitz, 1984). Growth and reproduction in *Daphnia magna* were also stimulated by water conditioned with crowded conspecifics (Burns, 1995).

By contrast, the negative effect of physical interference due to crowding on growth and reproduction in cladocerans has been shown (Guisande, 1993; Gosser and Ratte, 1994; Lee and Ban, 1999). By using a flow-through technique, which maintains a sufficient food supply and minimizes the accumulation of metabolites released from the animals, Guisande (Guisande, 1993) suggested that the negative effect of crowding on reproduction in *Daphnia magna* may be induced by physical contact between individuals. Lee and Ban (Lee and Ban, 1999) adopted a similar method, and suggested that *Simocephalus vetulus* might respond to neighboring individuals in crowded situations with a reduction in growth and reproduction.

Unfortunately, in these experiments, it was difficult to completely eliminate the effects of metabolites released by the animals, because the threshold concentrations that could induce negative effects and the effective exposure durations are still unknown (Guisande, 1993; Lee and Ban, 1999).

In the present study we tried to separate three crowding effects, namely, food shortage, accumulation of metabolites or infochemicals, i.e. information-conveying chemicals (Dicke and Sabelis, 1988), from crowded animals, and physical contact. We evaluated physical interference without food depression and chemically mediated effects by using flow-through chambers connecting crowded and non-crowded treatments. A flow-through system maintains a constant food supply and prevents accumulation of the metabolites released by experimental animals. If effective infochemicals are released by crowded animals, a single animal in a vessel connected to that of the crowded animals should consequently be exposed to the infochemicals and respond in the same manner as the crowded animals. Therefore, if the responses in the animals in crowded and non-crowded conditions differ significantly, they must be caused exclusively by physical interference.

In most previous studies, changes in demographic parameters in cladocerans under crowded conditions were determined for just one generation. It has been shown for several *Daphnia* species that...
maternal investment for reproduction can change life history strategies in the next generation; females born larger grow faster and mature to larger sizes than those born smaller (Tessier and Consolatti, 1989; Lampert, 1993; Ebert, 1994). Under crowded conditions, larger neonates have been shown to be produced in several cladocerans, although the number of the eggs released declines (Guisande, 1993; Cleuvers et al., 1997; Lee and Ban, 1999). Such reproductive responses to crowding may influence the fitness of the next generation through a shift of life history strategy. Cleuvers et al. (Cleuvers et al., 1997) investigated changes in life history traits in *Daphnia magna* by crowding throughout two consecutive generations, and showed that the effects of crowding were enhanced during the second generation. However, information on such maternal effects on crowding responses is still limited.

We, therefore, also examined growth and reproduction rates and life spans in a clone of *Daphnia pulex* reared under crowded and non-crowded conditions throughout two consecutive generations (mothers and daughters), in order to clarify the effects of physical interference due to crowding on life history traits. Again a modified flow-through system was used to avoid the accumulation of metabolites and to eliminate the potential effect of infochemicals.

We conducted the experiments at extremely different densities for the following reasons. Responses to crowding have been shown to be induced by a few surrounding individuals in laboratory experiments in several species of daphnids (Matveev, 1993; Goser and Ratte, 1994; Ban et al., 2008). When three to four *Daphnia carinata* are reared in an experimental container their clutch sizes are reduced to a half that of individually reared animals (Matveev, 1993). Furthermore, a reduction in clutch size occurs in *Daphnia magna* when just two individuals exist together in a container (Goser and Ratte, 1994). In our previous study, the ingestion rate of *Daphnia pulex* also declined even with three individuals per container (Ban et al., 2008). This indicates that the potential value of demographic parameters independent of daphniid density should be measured in a single individual in an experimental container. However, patchiness or aggregation behavior is a well-known phenomena for a single individual in an experimental container. Therefore, we conducted the experiments with a single individual in an experimental container as a non-crowding treatment, and with 20 and 40 individuals as the crowding treatments (equivalent to 400 and 800 ind. l⁻¹, respectively).

**MATERIALS AND METHODS**

*Daphnia pulex* Leydig was collected from an experimental pond at the Faculty of Fisheries, Hokkaido University, Japan. *Daphnia pulex* stock culture was established for more than two years prior to the experiments from a single female maintained in a 900 ml jar filled with glass-fiber-filtered (Whatman GF/C) and autoclaved-aged tap water (ATW) and food suspension of *Chlamydomonas reinhardtii* (strain no. C-9, Institute of Applied Microbiology, Tokyo, Japan) at 10⁵ cells ml⁻¹, which is equivalent to 4.1 μgC ml⁻¹ and far above the incipient limiting food concentration (0.5 μg C ml⁻¹) (Lampert and Schober, 1980), at 20°C, 12 h:12 h L:D. The population density of the stock culture was less than 50 daphniids l⁻¹ to avoid the influence of crowding. *Chlamydomonas reinhardtii* was grown on C medium (Ichimura, 1971) at 20°C, 12 h:12 h L:D (∼22 μmol m⁻² s⁻¹). The cells were centrifuged at 4000r.p.m. for 10 min at exponential growth phase, and then washed with ATW. Cell concentrations were calculated using a hemocytometer (Erma, Tokyo, Japan) before use as food.

All experiments were conducted with a flow-through system in order to maintain a constant food concentration and to avoid the accumulation of metabolites released by the animals (Fig. 1). Polystyrene tissue culture flasks (50 ml; Corning, NY, USA) were used in all experiments. Three flasks were connected in series through a 2 cm² x 2 cm window fitted with a net of 20 μm mesh, which allowed water and algal cells, but not daphnids, to flow freely to the other flasks. Food medium, consisting of ATW and *Chlamydomonas reinhardtii* at 10⁶ cells ml⁻¹, was placed in a stock reservoir (21 glass beaker), aerated to prevent depletion of oxygen during the dark period and settlement of the algal cells, and allowed to flow using gravity through a Tygon tube (inner diameter, 2 mm; Saint-Gobain Performance Plastics, Paris, France) into one end of the serially connected flasks. The food medium flowed through the 20-μm mesh into the middle flask, and then the last flask, and finally overflowed through a siphon. In order to provide a constant supply of sufficient food for the growth and reproduction of *Daphnia pulex* in the experimental flasks, the flow rate was maintained constant at 70 ml h⁻¹. This flow rate is sufficient to keep the algal concentration constant, because the filtering rates of *Daphnia pulex* are <20 ml daphniid⁻¹ day⁻¹ at 10⁶ cells ml⁻¹ of *Scenedesmus* spp. 

(Horton et al., 1979).

![Fig. 1. Schematic diagram of the flow-through system used in the experiments.](image-url)
In the experiments to determine the growth and reproduction of *Daphnia pulex* at different population densities, 1, 20 and 40 neonates, born within 24 h from the stock cultures were placed in each of the three serially connected experimental flasks. The neonates synchronously released from three or more clutches were used, because numerous neonates (>180 ind.) were needed for an experiment. TriPLICATE trials were conducted until 13 days from the start of the experiment. Since the medium flowed freely between the flasks, the daphniid in the single-treatment flask would be exposed to metabolites or infochemicals from the crowding treatments. Furthermore, each single treatment flask was placed in a different position among each set of the serially connected flasks in the triplicate trials, namely, 40–20–1, 1–40–20, and 20–1–40 from the inflow to outflow flasks, to ensure exclusion of the effects of chemicals from the crowded treatments. If chemically mediated effects were more influential than physical interference, no significant differences between the treatments would be found.

The growth and reproduction of the animals were checked every day, and each experimental flask was simultaneously replaced by a new flask. Each experimental animal was placed on a glass slide and body length, excluding the tail spine, was measured with an ocular micrometer attached to a dissecting microscope (Nikon, Tokyo, Japan) at ×40 magnification (sensitivity, 0.01 mm) under an indirect light source using a white-fluorescent tube. Immediately after measurements were taken, each animal was transferred to a new flask filled with fresh food medium. All procedures were performed within 60 s to avoid drying stress. After the animals matured, the number of eggs or embryos in the brood pouch and body size were measured with an ocular micrometer attached to a dissecting microscope (Nikon, Tokyo, Japan) at ×40 magnification (sensitivity, 0.01 mm) under an indirect light source using a white-fluorescent tube. Immediately after measurements were taken, each animal was transferred to a new flask filled with fresh food medium. All procedures were performed within 60 s to avoid drying stress. After the animals matured, the number of eggs or embryos in the brood pouch and body length, excluding the tail spine, was measured with the same procedure described above. Then, the animal was rinsed with distilled water and placed onto a pre-weighed aluminum pan. After it was dried at 60°C for 24 h, the dry mass was measured with an electro-balance (Mettler-Toledo International, Greifensee, Switzerland; sensitivity; 1 µg). Triplicate measurements were made for each treatment, and the average values were used to follow regression analysis. Then, the weight-specific reproduction rate (WSR) was calculated using the equation:

\[
\text{WSR} = C \frac{W_{\text{neo}}}{W_{\text{mother}}} t^{-1},
\]

where \(C\) is the clutch size at each brood, \(t\) is time in days of interbrood duration, and \(W_{\text{neo}}\) and \(W_{\text{mother}}\) are body dry masses of neonates and mothers, respectively.

Prior to testing differences among the treatments, i.e., density, the potential effect of infochemicals released from crowded daphniids was evaluated using the data set from the single-treatment daphniids in the first generation, to ensure an independence from chemically mediated effects. The effect of the position of the single-treatment flask in the flow-through chamber on body size and clutch size were tested by an analysis of covariance with the individual’s age and body size as covariates. The average values of each parameter (e.g. body size, clutch size, neonate size and number of neonates) in each replicate were calculated for the two crowding treatments. Then differences in body size and each life history parameter among the treatments were tested by a one-way analysis of variance (ANOVA). A multiple comparisons test was then conducted using Fisher’s least-significant-difference method if the results of the ANOVA identified significant differences. The differences in each life history parameter

Table 1. Homogeneity of the regression slopes between body size of *Daphnia pulex* reared singly and its age or between its clutch size and body size among the position of the single treatment flasks in the flow-through chambers

| Variable | Source | d.f. | SS   | MS   | F    | P    |
|----------|--------|------|------|------|------|------|
| Body size | Position × age | 2    | 0.042| 0.021| 0.940| 0.399|
|          | Residual | 36   | 0.803| 0.022|      |      |
| Clutch size | Position × body size | 2    | 17.19| 8.596| 1.239| 0.405|
|          | Residual | 3    | 20.81| 6.936|      |      |

d.f., degrees of freedom; SS, sum of squares; MS, mean square.

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Physical interference in daphniids between the two generations (mother and daughter) were tested with t-tests. All statistical tests were performed using StatView software (SAS Institute, Cary, NC, USA).

RESULTS

Evaluation for potential effect of infochemicals
To ensure an independence from chemically mediated effects, the potential effect of infochemicals that would be released by the crowded Daphnia pulex was examined. In the first generation, body size and clutch size of Daphnia pulex in the single treatment linearly increased with its age and body size (see Fig.2A, Fig.4A; \( r^2 > 0.9 \)), and slopes of the linear regression lines in body size and clutch size were not significantly different among the replications, i.e. position of the single-treatment flasks in the flow-through chamber (Table1). Since linearity of regressions and homogeneity of the regression slopes were confirmed, then differences in body size and clutch size between the position of the single-treatment flasks in the flow-through chamber could be tested with analysis of covariance (ANCOVA). ANCOVA shows no significant difference in both body size and clutch size among the position (Table2). This means that neither growth nor reproduction in Daphnia pulex tested was influenced by the position of the flasks, and therefore that effect of infochemicals from the crowded samples does not have to be considered.

Somatic growth
In the first generation, the growth curves of Daphnia pulex were similar in all the treatments until day 3 (Fig. 2A) and body length was not significantly different between the treatments. After that, however, the animals grew more slowly in crowded treatments, that is, in the 20 and 40 ind. flask\(^{-1}\). At day 4, body lengths in the two crowded treatments were already significantly less compared with single individuals. The body lengths of primiparous females, which were the first to bear eggs in their brood pouches, were not significantly different in the two crowded treatments, but the body lengths of older females at day 13 were significantly shorter in the 40 ind. flask\(^{-1}\) compared with those in the 20 ind. flask\(^{-1}\) treatments (Table 3).

In the second generation, the growth pattern of Daphnia pulex was quite different between the single ind. flask\(^{-1}\) and the two crowded treatments, even during early life stages, whereas it was similar between the two crowded treatments throughout the study period (Fig. 2B). Although the bodies of the newborns were larger in the two crowded treatments than those of the single ind. flask\(^{-1}\), as described below, they grew more slowly (Fig. 2B). Moreover, body size in the crowded treatments was significantly smaller than in the single ind. flask\(^{-1}\), even at day 2. Body length increments during the 2 days were 2.1-fold in the single ind. flask\(^{-1}\) and 1.3-fold in the two crowded treatments.

The growth of Daphnia pulex in the single ind. flask\(^{-1}\) was faster in the second generation than the first generation until day 6, but was slower thereafter (Fig. 3). The body lengths reached a plateau after day 6 in the single ind. flask\(^{-1}\); however, no such plateau occurred in either of the crowded treatments (see Fig. 2B). Although mean body size of primiparous females was slightly larger in all

Table 2. Results of analysis of covariance on body size and clutch size in Daphnia pulex among position of the single-treatment flasks in the flow-through chambers with its age and body size as covariate

| Variable      | Source     | d.f. | SS   | MS   | F    | P      |
|---------------|------------|------|------|------|------|--------|
| Body size     | Age        | 1    | 13.26| 13.262| 596.2| <0.0001|
|               | Position   | 2    | 0.822| 0.041| 1.842| 0.172  |
|               | Residual   | 38   | 0.845| 0.022|      |        |
| Clutch size   | Body size  | 1    | 236  | 236  | 31.05| 0.003  |
|               | Position   | 2    | 18   | 9.027| 1.188| 0.378  |
|               | Residual   | 5    | 38   | 7.6  |      |        |

d.f., degrees of freedom; SS, sum of squares; MS, mean square.
Table 3. Life history traits of *Daphnia pulex* reared at densities of 1, 20 and 40 individuals in a 50-ml flask in the first and second generations, and the results of ANOVA among the treatments and multiple comparison tests by Fisher’s PLSD.

| Life history traits | Generation | Density | ANOVA | Multiple comparison by Fisher’s PLSD*** |
|---------------------|------------|---------|-------|----------------------------------------|
| Body length of primiparous female (mm) | First | 1 Ind.† | 20 Ind.† | 40 Ind.† | d.f. | F | 1 ind. | 20 ind. | 40 ind. | 1 ind. | 20 ind. | 40 ind. |
|                      | Second | 1.89±0.14 | 1.67±0.05 | 1.56±0.12 | 2, 6 | 7.9* | 1 ind. | 20 ind. | 40 ind. |
|                      |         | 1.75±0.09 | 1.44±0.10 | 1.33±0.15 | 2, 6 | 10.8* | 1 ind. | 20 ind. | 40 ind. |
|                      |         | 1.52 | 3.69* | 2.07 |         |       |       |       |       |
| Body length of female at 13 days (mm) | First | 1.92±0.02 | 1.81±0.06 | 2, 6 | 137.3** | 1 ind. | 20 ind. | 40 ind. |
|                      | Second | 2.00±0.10 | 1.78±0.03 | 1.72±0.01 | 2, 6 | 17.9** | 1 ind. | 20 ind. | 40 ind. |
|                      |         | 6.05* | 6.15** | 2.81* |         |       |       |       |       |
| First appearance of primiparous females (days) | First | 7.0±0.0 | 7.0±0.0 | 6.7±0.6 | 2, 6 | 1.0 |       |       |       |
|                      | Second | 4.7±1.6 | 5.0±1.0 | 4.3±1.5 | 2, 6 | 0.2 |       |       |       |
|                      |         | 3.5* | 3.46* | 2.48 |         |       |       |       |       |
| First appearance of newborn neonates (days) | First | 9.0±0.0 | 9.0±0.0 | 9.0±0.0 | 2, 6 | 1.0 |       |       |       |
|                      | Second | 6.7±1.2 | 7.0±1.0 | 5.7±0.6 | 2, 6 | 1.6 |       |       |       |
|                      |         | 3.50* | 3.46* | 10.00** |         |       |       |       |       |
| Cumulative number of neonates after 13 days (per female) | First | 16.0±8.9 | 4.9±1.0 | 2.5±1.8 | 2, 6 | 5.6* | 1 ind. | 20 ind. | 40 ind. |
|                      | Second | 19.3±1.2 | 3.1±0.3 | 2.1±1.0 | 2, 6 | 346.2** | 1 ind. | 20 ind. | 40 ind. |
|                      |         | –0.64 | 2.93* | 0.29 |         |       |       |       |       |

*Significant level at *P*<0.05, **P*<0.01. ***Significant differences (P*<0.05) are not underscored by the same line.
†Values are means ± s.d.

In the first generation, primiparous females simultaneously appeared at day 7 in all treatments, and then all released their neonates at day 9 (Table 3). The average number of neonates first released by a female in the single ind. flask–1 was six per female, whereas it was less than one per female in the two crowded treatments (Fig.2A). Neonates were released twice by day 13 in all treatments, but females laid their eggs three times during the period. The cumulative numbers of neonates released in the single, 20 and 40 ind. flask–1 during the study period were 16.0, 4.9 and 2.5 per female, respectively. The number of neonates was significantly greater in the single ind. flask–1 than that in the two crowded treatments, but it was not significantly different between the two crowded treatments (Table 3). In the second generation, primiparous females appeared at days 4–6 in all treatments, and it was 1–3 days (average, 2 days) earlier than in the previous generation (Table 3). The average number of neonates first released by females in the single ind. flask–1 treatment was 5.5 per female (Fig.2B). The cumulative numbers of neonates released in the single, 20 and 40 ind. flasks–1 treatments during the study period were 19.3, 3.1 and 2.1, respectively (Table 3). These were almost the same as for the previous generation.

Clutch sizes were always larger in the single ind. flask–1 than the two crowded treatments in the first generation (Table 4). The clutch sizes in the single ind. flask–1 treatment increased linearly with body length from 8 to 19 eggs per female (Fig.4A). The sizes of the second and third clutches of the second generation in the single ind. flask–1 treatment were smaller than those of the first generation, but this was probably due to a lack of large females (greater than 2.00 mm; Fig.4B). In considering the relationship between parental body length and clutch size in the first generation, the clutch sizes in the second generation corresponded to the values predicted by the body lengths of the parents. Clutch sizes in the two crowded treatments decreased slightly with increasing body size, and were much smaller than five eggs per female in both generations.

The average body sizes of the neonates released by females in the first generation, in both the first and second clutch, were less than 0.65 mm in the single ind. flask–1 treatment, whereas they were larger than 0.68 mm in the two crowded treatments (Fig.5A). The difference among the treatments was statistically significant (ANOVA, d.f.=2, 15, F=14.38, *P*<0.01). Furthermore, there was no significant difference between the treatments in the second generation (ANOVA, d.f.=2, 22, F=0.405, *P*=0.67). The body sizes of the neonates released by the females in the 20 and 40 ind. flask–1 treatments were not significantly different from those in the previous generation (t-test, d.f.=12 and 13, *t*=-0.471 and –1.843, *P*=0.65 and 0.09, respectively), whereas those in the single ind. flask–1 treatment were variable and significantly larger.
than those in the previous generation (t-test, d.f.=12, t=-2.53, P<0.05).

Before calculating weight-specific reproduction rates (WSR) of *Daphnia pulex*, mass–length equations were established in each treatment using the flow-through system, to calculate body dry mass from body length of the experimental animals:

\[
\ln W = 0.774 \ln L + 2.903
\]

\(n=3, r^2=0.961, P<0.05\) at 1 ind. flask\(^{-1}\),

\[
\ln W_{20} = 0.456 \ln L_{20} + 2.802
\]

\(n=4, r^2=0.862, P<0.01\) at 20 ind. flask\(^{-1}\) and

\[
\ln W_{40} = 0.538 \ln L_{40} + 2.801
\]

\(n=4, r^2=0.916, P<0.01\) at 40 ind. flask\(^{-1}\), where \(W\) and \(L\) are body dry mass and body length of the animals, respectively. Although ANCOVA showed no significant difference among the treatments (d.f.=2, F=2.45, P=0.16), we used appropriate equations in each density for precise prediction.

WSRs of *Daphnia pulex* in the first generation were 1.71, 1.52 and 1.31 day\(^{-1}\) in the single, 20 and 40 ind. flask\(^{-1}\) treatments, respectively, at the first clutch, and differences between the treatments were not statistically significant (Table 5). WSR at the second clutch was 1.73 day\(^{-1}\) in the single ind. flask\(^{-1}\) and apparently decreased with increasing population density, although the differences between the treatments were not significant due to large variances. WSRs in the two crowded treatments were significantly different between the first and second clutches (t-tests, d.f.=4, t>3.4, P<0.05) but those in the single ind. flask\(^{-1}\) treatment were not (t-tests, d.f.=4, t=-0.04, P=0.97). In the second generation, although ANOVA could be calculated for the second clutch only because of limited datasets for the statistical tests at the first and third clutches, WSRs in the single ind. flask\(^{-1}\) treatment were larger than in the crowded flasks for all clutches (Table 5). Similar to the trend in the first generation, WSRs in the two crowded treatments were greater in the first clutch than in the second and third clutches.

**Ephippial egg production**

Six ephippial eggs (i.e. diapausing eggs) were produced in each crowded treatment 12 days after the start of the experiment, although this was only 2% of the cumulative number of neonates released until the end of the experiment (i.e. day 13). However, no ephippial eggs were produced by the single ind. flask\(^{-1}\) during the study period (Table 6). In the second generation, females reared the highest density (the 40 ind. flask\(^{-1}\)) produced 42 ephippial eggs after day 7. This represented 16.5% of the cumulative number of neonates. No males were produced in any treatments throughout the study period. This might be related to obligate parthenogenesis; females can produce diapausing eggs asexually (i.e. without male production) (Hebert, 1987), though cladocerans usually produce male offspring on detecting some environmental cues for forthcoming catastrophic events, such as photoperiod and crowding, and then produce ephippial (i.e. diapausing) eggs sexually (Hebert, 1978).

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### Table 4. Clutch size (eggs per female) of *Daphnia pulex* reared at densities of 1, 20 and 40 individuals per 50-ml flask in the first and second generations, and results of ANOVA among the treatments and multiple comparison tests by Fisher’s PLSD

| Generation | Clutch sequence | 1 Ind. | 20 Ind. | 40 Ind. | ANOVA | Multiple comparison by Fisher’s PLSD*** |
|------------|----------------|--------|---------|---------|-------|---------------------------------------|
| First      | 1              | 8.0±1.0| 4.5±0.6 | 4.0±1.2 | 2.6   | 14.6**                               |
|            | 2              | 13.0±6.9| 3.6±0.4 | 2.5±0.8 | 2.6   | 6.2*                                 |
|            | 3              | 18.8±2.8| 3.3±0.0 | 2.1±0.5 | 2.6   | 99.0**                               |
| Second     | 1              | 9.0±1.4| 3.3±0.4 | 3.3±1.1 | -     |                                       |
|            | 2              | 10.1±4.6| 2.7±0.2 | 2.5±0.3 | 2.6   | 7.9*                                 |
|            | 3              | 8.8±1.6| 2.5±0.2 | 1.9±0.4 | 2.5   | 53.8**                               |

*Significant level at P<0.05. **P<0.01.

***Significant differences (P<0.05) are not underscored by the same line.

†Values are means ± s.d.

Dashes denote that ANOVA was not calculated because of insufficient datasets.

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**Fig. 4. Relationship between clutch size and body size in *Daphnia pulex* at three population densities in the first (A) and second (B) generations. Vertical and horizontal bars represent standard deviations.**
DISCUSSION

In this study, *Daphnia pulex* grew faster and matured earlier in the second generation than in the first in all density treatments. It has been shown that offspring born from an earlier clutch reproduce earlier in several cladocerans (Ebert, 1991; Ebert, 1994). We used neonates from the third or later clutches for the first generation experiments in order to prepare numerous neonates for the experiments, whereas we used the first clutch for the second generation experiments. Therefore, earlier maturation of the females in the second generation compared with the first may be related to the earlier age that females released the neonates used in the experiments.

In this study, although the flow-through system maintained a sufficient food supply and prevented the accumulation of metabolites released by daphniids, *Daphnia pulex* grew more slowly and produced less offspring in both crowded conditions compared with the non-crowded treatment throughout the two consecutive generations. Because we connected single-treatment flasks to crowded flasks, infochemicals released by crowded animals could also affect single animals. Therefore, the single animals could respond in the same manner as the crowded animals, and no significant difference should be found between single and crowded treatments. However, the results of ANCOVA on body size and clutch size of *Daphnia pulex* reared singly showed no potential effect of infochemicals from crowded animals on both growth and reproduction. Besides, in most cases, the results of ANOVA on each demographic parameter did differ significantly among the density treatments. These results suggested that chemically mediated effects were not influential on the shifts in demographic parameters observed in this study. Additionally, in our recent study using the same clone of *Daphnia pulex*, the ingestion rate was suppressed by a high population density but not by water preconditioned with crowded conspecifics for 3 days (Ban et al., 2008). This evidence demonstrates that the negative effects of crowding on several demographic parameters in *Daphnia pulex* are exclusively induced by physical interference among the individuals, such as physical contact between neighboring individuals or pulses generated by the beating of the thoracic limbs of other individuals.

There is ample evidence to demonstrate that physical interference affects feeding, reproduction and behavior in other zooplankton and arthropods. A reduction in egg numbers per ovisac in the marine harpacticoid *Amphiasoides* sp. was found to be directly due to a high density of animals, regardless of food levels, rather than the conditioned water of a crowded culture (Walker, 1979). Miralto et al. (Miralto et al., 1996) showed that egg production rates in the marine calanoid *Centropages typicus* reduced in conjunction with increasing animal density, but was not modified by metabolites from the copepods themselves, again suggesting that physical disturbance and an increasing number of encounters were more important for the detection of crowding than chemical communication. In larvae of the mosquito *Culex sitiens*, feeding was inhibited by mechanical

**Table 5. Weight specific reproduction rates (per day) of *Daphnia pulex* reared at densities of 1, 20 and 40 individuals per 50-ml flask in the first and second generations, and results of ANOVA among the treatments and multiple comparison tests by Fisher’s PLSD**

| Generation | Clutch sequence | 1 Ind. | 20 Ind. | 40 Ind. | d.f. | F   | Multiple comparison by Fisher’s PLSD** |
|------------|-----------------|--------|---------|---------|------|-----|--------------------------------------|
| First      | 1               | 1.71±0.14 | 1.52±0.20 | 1.31±0.37 | 2, 6 | 1.87 |
|            | 2               | 1.73±0.91 | 0.77±0.10 | 0.50±0.15 | 2, 6 | 4.39 |
| Second     | 1               | 2.13±0.20 | 1.12±0.11 | 1.12±0.37 | 2, 3 | –   |
|            | 2               | 1.51±0.58 | 0.62±0.05 | 0.53±0.08 | 2, 6 | 7.51* | 1 ind. | 20 ind. | 40 ind. |
|            | 3               | 1.41 | 0.54±0.03 | 0.39±0.07 | 2, 3 | –   |

*Significant level at *P*<0.05.

**Significant differences (*P*<0.05) are not underscored by the same line.

Values are means ± s.d.

Dashes denote that ANOVA was not calculated because of insufficient datasets.
interference due to overcrowding (Roberts, 1998). In terrestrial insects, such as *Locust migratoria*, there are extensive studies on behavioral changes in response to population density, and it has been shown that tactile contact, such as stimulation with fine wires, which simulates physical contact due to crowding, can induce changes from solitary to gregarious behavior in phase polyphenism (Tanaka, 2006).

In this study, we showed the negative effects of crowding on growth and reproduction in *Daphnia pulex*, even with a sufficient food supply. As shown in the literature, crowding by conspecifics reduces adult growth and egg production rates in *Daphnia pulex* (Nishikawa and Ban, 1998) and *Simocephalus vetulus* (Lee and Ban, 1999), clutch size in *Daphnia pulex* (Fitzsimmons and Innes, 2006), *Daphnia hyalina* (Seitz, 1984), *Daphnia carinata* (Matveev, 1993) and *Daphnia magna* (Guisande, 1993) given excess food. What causes such negative effects on demographic parameters? Several studies showed that crowding or high population density reduced feeding in cladocerans (Hayward and Gallup, 1976; Peters and Downing, 1984; Helgen, 1987). Since a reduction in the feeding of an animal consequently lowers its growth and reproduction, these negative effects may be explained by a reduction in feeding due to crowding (Matveev, 1993; Burns, 1995). However, the responses shown in body size and the timing of maturation in cladocerans seem to be different between females reared in food-limited and crowded conditions. When food concentration is low, several daphniids grow slowly and retard their maturity until the body size reaches a certain level (*Daphnia galeata* (Urzebe, 1988); *Daphnia pulex* (Riessen and Sprules, 1990); *Daphnia hyalina* (Guisande and Gliwicz, 1992); *Daphnia magna* (Ebert, 1994)). By contrast, cladocerans in crowded conditions with an adequate food supply mature at the same time but with smaller bodies than those in non-crowded conditions (Nishikawa and Ban, 1998; Lee and Ban, 1999) (and this study). Thus, *Daphnia* may respond differently to food shortages and crowding conditions. This evidence suggests that the negative effects on growth and reproduction due to crowding cannot be adequately explained by a food shortage.

There is very limited information on crowding effects based on demographic parameters throughout the whole life history, that is, from hatching to adulthood, in cladocerans (Lee and Ban, 1999). Nishikawa and Ban (Nishikawa and Ban, 1998) found that the effect of crowding on growth in *Daphnia pulex* could change ontogenetically and occurred after maturation. In *Simocephalus vetulus*, the growth of an adult is lowered by crowding, but that of juvenile instars is not (Lee and Ban, 1999). In daphniids, it has been shown that each reproductive event, in other words, the production of the clutch, takes in total three instars (Zaffagnini, 1987; Bradley et al., 1991; Ebert and Yampolsky, 1992). Generally, oogenesis and differentiation of oocytes from nurse cells occurs in the first of these instars. The provision of oocytes with maternal reserves and yolk formation occur during the second instar, and eggs are then deposited into a brood pouch at the beginning of the third instar. In this study, the growth curves of *Daphnia pulex* in the first generation differed between treatments after day 4, and egg-bearing females first appeared at day 7 in all treatment conditions. Oogenesis in daphniids is considered to start at day 4 by tracing the molt of the daphniids in a single-individual treatment. In the second generation, oogenesis also seems to start on the same day (day 2) when growth is reduced in the crowded conditions because reproduction started 2 days earlier than that in the first generation. Therefore, the negative effect of crowding on somatic growth in *Daphnia pulex* seems to occur after oogenesis starts. These findings imply that lowered growth rates in crowded conditions are due to increased reproductive investment (Lee and Ban, 1999).

Larger neonates were produced during the two crowded treatments during the first generation of this study, compared with the non-crowded treatment. The same results have been shown in *Daphnia magna* (Guisande, 1993; Cleuvers et al., 1997) and *Simocephalus vetulus* (Lee and Ban, 1999). Also in *Daphnia hyalina* and *Daphnia galeata*, larger eggs are laid by females raised in crowded conditions (Burns, 1995). Such larger neonates have a greater lipid content (Cleuvers et al., 1997) and survive longer, even under severe food shortages (Tessier and Consolatti, 1989; Gliwicz and Guisande, 1992; Cleuvers et al., 1997). The rapid increase in daphniids after a spring bloom of edible algae is often responsible for an abrupt decline of the algal biomass as a result of a high filtering rate of the surrounding water by the daphniids (e.g. Lampert et al., 1986). The production of large neonates by females that detect crowded situations would be advantageous for survival under forthcoming severe environmental food conditions (Cleuvers et al., 1997).

Although egg production rates decreased with increasing animal density in *Simocephalus vetulus*, weight-specific reproduction rates (*WSRs*) in crowded animals were higher than those in non-crowded animals because of the production of larger neonates (Lee and Ban, 1999). This implies that crowded *Simocephalus vetulus* may invest more energy in reproduction than in somatic growth, and thus produce larger offspring (Lee and Ban, 1999). Also in this study, *WSRs* in *Daphnia pulex* were not significantly different between crowded and non-crowded treatments in the first clutch, despite the smaller clutch sizes in crowded flasks, although most remaining cases could not be analyzed because of insufficient datasets. This suggests that during at least early adult instar, *Daphnia pulex* might invest more energy in reproduction than somatic growth and produce larger offspring in preparation for severe food shortages (Cleuvers et al., 1997; Lee and Ban, 1999). Therefore, a reduction in the growth rates in crowded *Daphnia pulex* may in part be explained by a trade-off between growth and reproduction.
WSRs of Daphnia pulex were lower in the second and third clutches than in the first in the two crowded treatments. This was caused by the extremely small clutch sizes in older animals under crowded conditions. It is not likely that such small clutch sizes were attributable to a loss of energy through a lowered per capita feeding rate caused by crowding. Such small clutch sizes in older females seem to be rather a unique response of the clone used in this study. In our previous study, using a different clone of Daphnia pulex, crowded females spawned more eggs when older rather than younger (Nishikawa and Ban, 1998). In Simocephalus vetulus, a similar positive correlation between clutch size and female age was found in all density treatments (Lee and Ban, 1999). Older Daphnia magna females have also been shown to spawn more eggs even under crowded conditions (Goser and Ratte, 1994; Clevers et al., 1997). The evidence indicates that a reduction in feeding due to crowding does not necessarily cause a diminution of clutch size in older animals. Since inter-genotype variation in reproductive responses to crowding has been shown in Daphnia pulex (Fitzsimmons and Innes, 2006), smaller clutch sizes at older age under crowded conditions may be a reproductive feature of the clone used in this study, but is not attributable to food shortage. If the reproductive responses to crowded conditions adapt to forthcoming severe food conditions as described above, the reduction in offspring numbers may also be adaptive. Suppression of the population growth through reducing the number of offspring may also inhibit a food shortage. A lower production of large tolerable offspring would be expected to enhance their fitness under severe environmental food conditions.

Since diapause can avoid adverse environments temporarily, the production of diapausing eggs is advantageous for females where crowding factors are token stimuli indicating forthcoming deleterious environments, such as drought, accumulation of waste substances and food shortage. Carvalho and Hughes (Carvalho and Hughes, 1983) reported that diapausing egg production in Daphnia magna correlated positively with female density in a culture vessel with excess food. Recently, Fitzsimmons and Innes (Fitzsimmons and Innes, 2006) used six different clones to show that per capita diapausing egg production in Daphnia pulex was significantly higher for crowded daphniids than for individual animals. In this study, Daphnia pulex did produce diapausing eggs, but these represented only a small percentage of the cumulative number of parthenogenetic eggs. Variability in diapausing egg production among clones has been shown in Daphnia pulex (Innes and Singleton, 2000; Berg et al., 2001), Daphnia pulecara (Caceres and Tesser, 2004) and Daphnia magna (Carvalho and Hughes, 1983). Our Daphnia pulex clones are probably less sensitive to crowding in terms of diapausing egg production. Besides, we showed that females reared in the most crowded conditions in the second generation produced more diapausing eggs, suggesting that sensitivity to crowding in the offspring was enhanced when the mothers also experienced crowding.

In the present study, Daphnia pulex survived for less time in the most crowded condition than in non- and less-crowded conditions. There has been no evidence to suggest that crowding affects life span or survival in daphnids until now. In Drosophila melanogaster, a negative correlation has been shown between early reproduction and subsequent life expectancy (Zwaan et al., 1995; Tatar et al., 1996; Partridge et al., 1999). Recently, Silbermann and Tatar (Silbermann and Tatar, 2000) found antagonistic effects between early reproduction and later age-specific mortality through the production of heat shock proteins. Such a trade-off between early reproduction and longevity might be expected in crowded daphnids. In order to clarify this relationship, further studies for determining energy allocation throughout whole life stage under crowded conditions are required.

In conclusion, Daphnia pulex that experience physical interference as a result of crowding reach maturity with smaller body sizes and produce lower numbers of large tolerable offspring through a trade-off between growth and reproduction. They are capable of making resting propagules but do not survive longer.

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