Enhanced growth at low population density in *Daphnia*: the absence of crowding effects or relief from visual predation?

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SUMMARY

1. It has been suggested that chemical information from crowded populations of an animal such as *Daphnia* carries a cue indicating imminent food limitation, and we suggest that in the presence of fish kairomones, it may also convey a hint of the need to enhance antipredation defences.
2. We performed two-factorial experiments with *Daphnia* grown in flow-through plankton chambers in medium containing high levels of *Scenedesmus* food plus chemical information on either low or high population density levels and in the presence or absence of fish chemical cues (kairomones) and recorded (i) the effects on *Daphnia* growth rate and reproduction, and (ii) the effects on *Daphnia* depth selection. Further depth-selection experiments were performed to test the reaction of *Daphnia* to crowding information at different *Daphnia* concentrations and to test its effect on daytime and night-time depth selection by different *Daphnia* instars in the presence of kairomones.
3. The effects of crowding information alone (in the absence of kairomones) were weak and were not significantly strengthened by the addition of kairomones. The effects of kairomones alone (in the absence of crowding information) were much stronger and were increased by the presence of crowding chemicals: *Daphnia* selected greater depths in daylight (the later the instar and the larger its body size, the greater the depth), their body growth was slower and daily reproductive investment reduced, compared with *Daphnia* grown in the absence of crowding information. This suggested that crowding chemicals carry a cue indicating the need to invest more into antipredation defences.
4. The adaptive significance of these effects was confirmed by the differential vulnerability to predation of the *Daphnia* when offered as prey to live roach after being grown for 6 days either in the presence (higher vulnerability) or in the absence (lower vulnerability) of information on high density.
5. The strong interaction between crowding information and fish kairomones may be explained either as the reaction to a cue indicating impending food stress or as the reaction to a signal of increased predation risk. While the former scenario is already known from crowding studies, the latter is a novel idea that stems from the old concept of 'low-density anti-predation refuge'. The two scenarios are not mutually exclusive: each stems from the need to invest in survival rather than in growth and reproduction [Corrections were made to this paragraph after first online publication on 4 April 2012].

Keywords: crowding, defence costs, density dependence, predation risk, refuge

Introduction

Stress and other deleterious effects of crowding have been of interest to ecologists since this phenomenon was first reported by Frank (1952, 1957) and revisited by Folt & Goldman (1981). Their suggestion that the individual fitness of an animal such as *Daphnia* is lower at high population densities owing to stress resulting from an approaching decline in food levels has been pursued by others who have experimentally demonstrated that

information on high population density instigates a decline in ingestion rate (in *Daphnia Schoedleri* G.O. Sars – Hayward & Gallup, 1976; *Daphnia pulex* Leydig – Helgen, 1987; Ban *et al.*, 2008; or *Daphnia carinata* King & *Daphnia lumholtzi* G.O. Sars – Matveev, 1993), the rate of individual body growth (Burns, 1995, 2000), fecundity (Seitz, 1984; Lee & Ban, 1999; Rose, Warne & Lim, 2002) and the intrinsic rate of population increase (Boersma, 1999; Lürling *et al.*, 2003). The effects of these declines are reflected in the linear increase in the magnitude of individual growth limitation by resource and interference competition that stem from the logistic model of population growth up to the limits of carrying capacity (Fig. 1a).

Alternatively, the phenomenon of growth limitation at high population densities may be attributed to the need to activate antipredation defences at the cost of lost feeding opportunity and more energy allocated to defence when density increases. This is most likely within the range of low densities where the increase in individual risk of predation is most rapid, according to Holling's (1959) type III functional response (Fig. 1b). This fast increase in individual risk may be further accelerated by an aggregative (numerical) response of the predator in space, which is well known for planktivorous fish and their *Daphnia* prey (Gliwicz & Wrzosek, 2008).

While the dependence of individual growth limitation on density is linear up to the point where further growth becomes limited to that needed to sustain population abundance at the carrying capacity (Fig. 1a), the density dependence of individual risk has an initial phase of rapid increase followed by a gradual decline at high densities (Fig. 1b). Even though this decline would be moderated by the likely numerical responses of predators attracted to prey aggregations, it is difficult to judge the relative importance of the two scenarios because the values of the two factors, growth limitation and predation risk, are not necessarily additive at high population densities. They are additive when extremely low densities are compared with intermediate ones, around ½N, but not when the intermediate densities are compared with those near carrying capacity (Fig. 1a,b), when prey should benefit from the dilution of risk and predator confusion (Hamilton, 1971).

In comparison with the ideas of 'safety in a group' and 'high-density refuge', the notion of 'low-density refuge', based on an original idea of Rosenzweig & MacArthur (1963), has been less frequently discussed. The latter was first used to explain different densities of marine benthic molluscs by Lipcius & Hines (1986), Hines *et al.* (1997), Seitz *et al.* (2001) and Kuhlman & Hines (2005). Later, it was also suggested as a possible explanation for depth-selection behaviour in *Daphnia* (Gliwicz, Dawidowicz & Maszczyk,
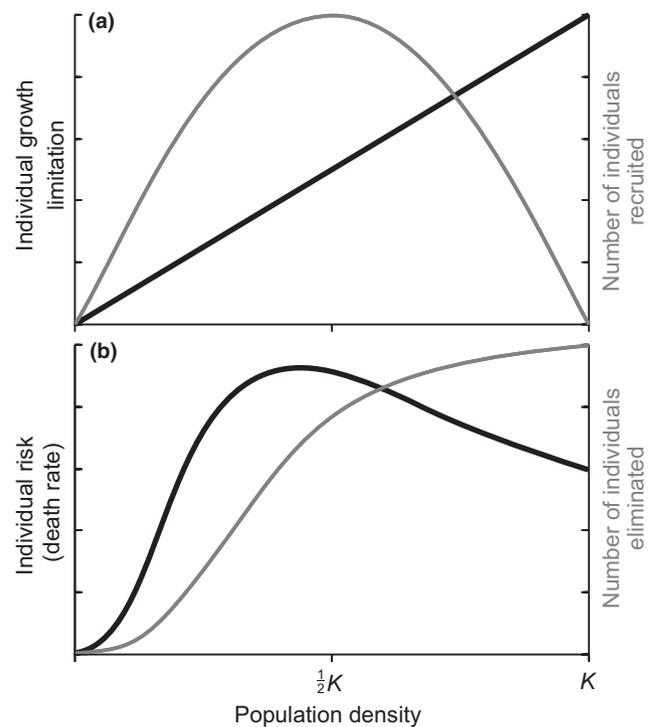

Fig. 1 Shift in the magnitude of individual growth limitation by competition (a) and the magnitude of individual risk to predation (b) along the prey population density spectrum within densities below the carrying capacity *K* (black lines), each taken as a function of the number of individuals recruited to (birth rate, *b*) or eliminated from (death rate, *d*) a population per unit of time (grey lines).The magnitude of individual growth limitation by the resource and the interference competition (black line in a) is an inversion of the function of per capita growth rate along the density spectrum that assures logistic growth of a population up to the carrying capacity level, *K* (number of individuals recruited per unit of time on population density level, grey line). The magnitude of individual risk to predation (black line in b) is derived from Holling's (1959) type III functional response depicting the change in a predator's capture rate (number of individuals eliminated, grey line) as captures per individual of the prey population (each value of the ordinate divided by the respective value of the abscissa, an inspiration from Young *et al.*, 1994). The range of low densities where prey benefit from a 'low-density antipredation refuge' (individual risk reduced to zero) can be extended to the right when it is assumed that the predator ignores the prey up to a critical prey density level that is equal to either the number of prey being able to find a refuge (Rosenzweig & MacArthur, 1963) or the number when the encounter rate of a given prey with the predator becomes too infrequent for it to be retained in the predator's diet (Gliwicz *et al.*, 2010).

2006), but this was done with no reference to the possibility of crowding effects as an alternate explanation of the different depths selected at low and high population densities and with no effort to assess the costs of antipredator defences.

In this study, we hypothesise that the reduced risk of predation at low population density should allow *Daphnia* prey to abandon costly defences induced by fish presence,

such as reduced rate of growth resulting in a decline in daily reproductive investment owing to smaller body size at maturity (Machaček, 1991; Stibor, 1992) and descent into deeper strata that are safer because of light reduction but cooler and less food proficient (De Meester *et al.*, 1999). The ability to assess whether the risk has been reduced as a result of low population density would allow *Daphnia* prey to increase their fitness by feeding closer to the surface with more food and at a higher temperature, and grow and reproduce as fast as is permitted by food levels alone, without the stress caused by the fear of predation that might be conveyed by crowding information. Hence, it would allow *Daphnia* to take advantage of 'the low-density antipredation refuge'. This optimum growth and reproduction would ensure high reproductive success, at least until high population density was restored and stress produced by a mixture of fish kairomones and crowding infochemicals caused antipredation defences to be switched on again.

The first hypothesis tested in this study is (i) that the growth and fecundity of *Daphnia* should decline in the presence of information on high density to a greater extent in the presence of fish kairomones than in their absence, suggesting that the effects of predation risk and the effects of crowding are additive, (ii) that this would be caused by the activation of antipredation defences, including descent to safer, less-well-illuminated strata of the water column, and (iii) that reduced growth and fecundity at high density would reflect the costs of antipredation defences that are paid by *Daphnia* at such density levels. Data to test this hypothesis were gathered from an experimental assessment of whether *Daphnia* at high population density grow more slowly (*growth rate*), reduce their daily reproductive investment (*reproduction*) and select greater daytime depths (*depth selection*).

The second hypothesis we test is that that the use of a 'low-density antipredation refuge', by giving up costly antipredator defences to increase fitness through faster growth and reproduction, may be a risky strategy. To check whether increased fitness at low density could increase mortality caused by unexpected predation, the vulnerability of *Daphnia* grown in the presence or absence of chemical information on high density was compared (*Vulnerability to fish predation*).

Methods

The approach

Daphnia were raised individually from the age of 0.5 days to the adult age of 5–7 days, when their body weight,

individual growth rate and fecundity were assessed. They were grown in a flow-through system in medium containing identical non-limiting levels of algal food (the initial food level of 1 mg C L^{-1}), but different information on population density, both in the presence and in the absence of fish kairomones. The diluted water from the crowded population could not contain alarm substance from dead *Daphnia* because alarm substance is known to be released only by live *Daphnia* when being captured by fish (Laforsch, Beccara & Tollrian, 2006) or crushed while in the process of alarm substance production (Pijanowska, 1997). In growth experiments, *Daphnia* were grown in a non-thermally stratified water column. In depth-selection experiments, they were grown in a thermally stratified water column, and the depth selected by each individual was registered every second hour. Chemical information related to high population density was prepared by diluting crowding water obtained from a mass *Daphnia* batch culture.

Experimental animals

All experiments were performed using the same clone of *Daphnia hyalina × galeata* (clone HG011) isolated from the hypolimnion of Lake Swiecajty in the Great Mazurian Lakes, a lake system known for high densities of planktivorous fish (smelt, *Osmerus eperlanus* L. and roach, *Rutilus rutilus* L.; Gliwicz, 2003; Gliwicz, Szymanska & Wrzosek, 2010). *Daphnia* were grown in the laboratory in batch cultures in 3-L glass jars at high *Scenedesmus obliquus* food levels (1 mg C L^{-1}) to produce thousands of age-synchronised individuals. The *Daphnia* cultures and all experiments were performed in media based on 0.45-μm Millipore mesh-filtered water from Lake Gora near Warsaw (52°27′ N, 20°46′ E) that had been conditioned by storage and aeration for 2 weeks to remove possible traces of information on crowding or fish predation (fish kairomones).

For both the growth rate assessment and depth-selection experiments, mass batch cultures of the same *Daphnia* clone were used as sources of water that had been chemically altered by a high density of conspecifics. Chemical information on population densities of 30–120 ind. L^{-1} used in the experimental system was prepared by diluting this crowding water with 0.45-μm Millipore mesh-filtered conditioned lake water. Mass *Daphnia* cultures of diverse body size and mixed-age individuals were started 48 h before each experiment and kept at a constant food level of 1 mg C L^{-1} and constant density of 240 *Daphnia* L^{-1} for the duration of the experiment by daily random removal of superabundant

Daphnia from the culture using a pipette. The crowding water from such a mass culture was first diluted with 0.45-μm Millipore mesh-filtered conditioned lake water to obtain chemical information equivalent to *Daphnia* population densities of 60, 90, 120 and 240 ind. L^{-1} (density in the mass culture being twice that desired, no crowding water was added to media in the control treatment), and then each was mixed with an equal volume of medium containing food (*S. obliquus*) and fish kairomones (information on predation risk from aquaria containing 2+ roach), to give a food level of 1 mg C L^{-1}, a kairomone concentration of 0.125 fish L^{-1}, and information on *Daphnia* densities of 30, 60, 90 or 120 ind. L^{-1} in the inflow to the separate experimental chambers. This level of kairomones has previously been tested on many *Daphnia* clones and found to be very effective as information on predation risk (De Meester *et al.*, 1999). The control low population density animals received the same levels of food and kairomones, but the medium was mixed 1 : 1 with 0.45-μm Millipore mesh-filtered conditioned lake water (without *Daphnia* information). For media free of information on predation risk, no kairomones were added to the low and high population density treatment water.

Experimental system

The same flow-through system was used for both the depth selection and growth assessment experiments (as used by Dawidowicz & Loose, 1992). This consisted of a set of 14, 28 or 49 glass flow-through tubular chambers (0.6 m long, 0.01 m internal diameter, 0.044 L volume) placed vertically in a transparent water bath that either ensured the same temperature (20 °C) throughout the water column (in growth assessments) or maintained thermal stratification within the chambers (depth-selection experiments), with 22 °C at the surface and 9 °C at the bottom, and a sharp (20–10 °C) thermocline between the depths of 0.25 and 0.40 m measured from the surface. Every chamber had an inflow at the top and an outflow at the bottom, with the latter covered by a 120-μm mesh net to retain the animals inside. Fine black horizontal lines marked on the front of the water bath were used to determine the depth of each *Daphnia*. The medium was supplied to the chambers at a constant flow rate via a single multichannel peristaltic pump fed from 5-L glass reservoirs that were mixed by air bubbling. The system was illuminated from above using a set of four halogen lamps (20 W, 12 V) shining through a frosted glass diffuser, to produce an illumination gradient from 8.4 μmol m^{-2} s^{-1} at the surface to 4.2 μmol m^{-2} s^{-1} at

the bottom, with a 12 : 12 L : D photoperiod. The media with chemical information on different population densities, with or without fish kairomones, were delivered to the flow-through chambers at the rate of 1.7 L d^{-1} per chamber. A grid of 120 infrared light-emitting diodes placed behind the transparent water bath containing the flow-through chambers could be turned on at night to read the depth of each *Daphnia* in darkness using a sensitive military night vision device: Monocular MU3 (Bumar, Warsaw, PL).

Assessing individual growth rate and fecundity

Assessments were made on *Daphnia* grown in flow-through chambers with chemical information on population densities of 1 and 60 ind. L^{-1}, as the control and the treatment, respectively. At the start of each experiment, all synchronised 0- to 12-h-old neonates were transferred randomly to the plankton apparatus, at one individual per chamber. To determine initial size, 20 individuals were collected, examined under a dissecting microscope to measure body length (from the upper edge of the eye to the base of the tail spine), rinsed in distilled water, then dried overnight at 60 °C and weighed using a sensitive Orion-Cahn C-35 balance (Thermo Electron Corp., Beverly, MA, U.S.A.). The same procedure was applied to 5- to 7-day-old adults, with each animal being collected from the chamber within an hour of oviposition. This was achieved by hourly visits to collect all adults with freshly deposited eggs in the brood cavities, so that they were all at roughly the same physiological state when their final weights were taken. This collection period lasted up to 48 h as there was substantial variability in the time of first reproduction despite the genetic identity and the same age of all replicate *Daphnia*. This variation was possibly due to the well-known flexibility in the decision whether eggs should be deposited in the present or in the subsequent instar (Ebert, 1994). Before being dried and weighed, an image of each adult was saved as a MultiScan (Miami, FL, U.S.A.) frozen screen to permit estimation of the *egg volume* by measuring the two axes (length and diameter) of each ellipsoidal egg in the brood chamber.

The integrated volume of all eggs in the first clutch permitted calculation of the daily reproductive investment, as the ratio of clutch volume to age at first reproduction, thus giving the rate of resource allocation for reproduction in each individual, irrespective of the highly variable age of first reproduction. In total, three experiments (G1–G3) were completed on *D. hyalina*, all with a two-factorial design (Table 1).

Table 1 Two-way ANOVA (F and P) for significance of the effect of fish kairomones (FK), the effect of crowding information (density of 60 *Daphnia* L^{-1}, CI) and the interaction between them (FK × CI) for different life history traits in growth experiments (the integrated data from three experiments G1–3). *, ** and *** denote differences significant at $P < 0.05$, $P < 0.005$ and $P < 0.0005$, respectively. These statistics do not take into consideration individual body length, which is considered in Fig. 3 and Table 3

Factor or interaction	Growth rate	AFR	SFR	Egg volume	Clutch size	Daily reproductive investment
FK	58.7***	90.7***	186.0***	41.8***	29.4***	37.4***
CI	2.6^{ns}	0.1^{ns}	5.2*	0.3^{ns}	2.8^{ns}	2.6^{ns}
FK × CI	8.4**	5.6*	5.3*	6.0*	0.2^{ns}	0.6^{ns}

Depth-selection experiments

Before being transferred to their individual flow-through plankton chambers, the synchronised 0- to 12-h-old *Daphnia* neonates were grown individually in batch cultures (each *Daphnia* in a 1 L glass jar) at high *Scenedesmus* food levels (1 mg C L^{-1}) either in the absence (low-density control) or in the presence of crowding information (30–120 ind. L^{-1}, high-density treatment). The 2-day-old juveniles were transferred to the flow-through chambers at one individual per chamber, and 6, 7 or 10 individuals per treatment, with a constant flow of medium as described above. The depth of each *Daphnia* was recorded every second hour during the 12 h of daylight, giving a total of seven observations per animal per day. A further five observations were made during the night using background infrared illumination and the military night vision device. A total of 13 experiments (D1–13) were completed, most in the presence of crowding information on 60 ind. L^{-1} (D4–10), including one with additional night-time depth estimations (D9, Table 4). A two-factorial design was employed in preliminary experiments with the experimental and control treatments performed in both the presence and absence of fish kairomones. However, no difference in depth selection was ever detected in the absence of kairomones; hence, only one experiment (D13) of those presented had such a design to confirm the preliminary results (Table 4). Experiment D13 was performed with different levels of crowding information (30, 60, 90 and 120 ind. L^{-1}).

Testing vulnerability to fish predation

In each of three replicate experiments (performed in February, April and May 2009), 600 synchronised 0- to 8-h-old *Daphnia* neonates were grown separately for 5 days in 200 mL batch cultures, in medium with the same high *Scenedesmus* food level (1 mg C L^{-1}) and the same concentration of fish kairomones (0.125 fish L^{-1}), which were replaced daily. For half of the *Daphnia*, the medium also contained information on high population density (60 *Daphnia* L^{-1}), while for the other half this information was absent. After 5 days, all 300 individuals from each group were mixed together in 5 L of filtered water from Lake Gora. A few minutes later, each group was gently transferred to the surface layer of a column of 200 L of filtered lake water in separate 'plankton towers' consisting of identical vertical non-transparent plastic (PVC) tubes of 0.45 m diameter and 1.73 m depth, where the final *Daphnia* density was set at 1.5 ind. L^{-1}. The day before, each column of water in these towers had been thermally stratified. The two towers, placed side by side, were illuminated from above with a 20-W lamp reflected from the white ceiling to produce an illumination gradient from 8 µmol m^{-2} s^{-1} at the surface to 0.08 µmol m^{-2} s^{-1} at the bottom of the water column - the subsurface illumination being well within the range suitable for roach predation activity (Gliwicz & Wrzosek, 2008). After a period of 20 min to permit acclimation and free distribution of the *Daphnia*, two 2-year-old roach of 60–70 mm body length, randomly selected from a stock tank, were added to each tower. These fish were allowed to feed for 30 min then removed, and the entire volume of 200 L of water from each tower was sieved through a 0.25-mm mesh plankton net. The retained *Daphnia* were counted to calculate the number harvested by the fish. This predation experiment was performed three times. The feeding duration of 30 min was selected after several preliminary experiments with durations of 20 and 60 min, which resulted in either too few *Daphnia* being eliminated by fish or left too few survivors to distinguish between treatments.

Results

Growth rate

The effect of fish kairomones was very strong and consistent, with the difference in growth rate being highly significant in each of the three experiments ($P < 0.05$, $P < 0.0005$, $P < 0.0005$, respectively) and in the integrated data (FK in Table 1), both in the absence and in the presence of crowding information (Fig. 2a). This difference
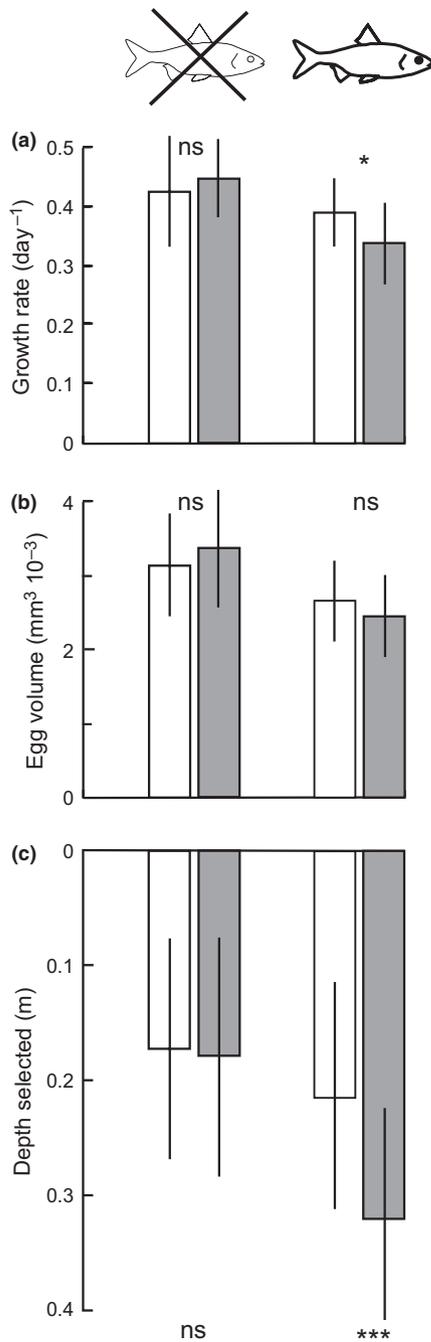
Fig. 2 Growth, per egg investment and depth-selection data for 4th and 5th instar *Daphnia* grown individually in the absence (empty bars) or in the presence (filled bars) of crowding information, and in the absence (no fish) or presence (fish) of fish kairomones (mean ± 1 SD): (a) daily body growth rate, and (b) per-offspring investment (egg volume) for 6- to 8-day-old *Daphnia* (each from pooled data from experiments G1-3, crowding information from a *Daphnia* density of 60 ind. L^{-1}); (c) daytime depth selected by *Daphnia* throughout their growth in the flow-through system, from the 4-day-old juveniles to the 6-day-old females (data from experiment D13, crowding information from *Daphnia* densities of 120 ind. L^{-1}, * and *** denote difference significant at $P < 0.05$ and $P < 0.0005$, respectively).

(nFK–FK) was found to be much greater in the presence (CI) than in the absence (nCI) of crowding information (Table 2), suggesting a strong interaction between the effects of the two factors.

The effect of crowding information was not found to be significant for the combined data from the two treatments (i.e. without and with the addition of kairomones; CI in Table 1), but the difference was significant for the data from the treatment with addition of kairomones (FK), where the growth rate was found to be reduced by 14% in the presence of crowding information (nCI–CI in Table 2, Fig. 2a).

The significant interaction between the effect of fish kairomones and the effect of crowding chemicals (FK × CL) was consistent from one experiment to another (Table 1). Judging from the effect expressed as the % difference in growth rate in Table 2, we calculated that the synergistic effect of both factors – fish kairomones (FK) and crowding information (CI) – on growth rate (nFK–FK, CI) was much greater (28.2%) than the sum of the % reduction produced by kairomones alone (13.6%, nFK–FK, nCI) and by crowding information alone (−3.6%, nCI–CI, nFK) (i.e. merely a third of this value; 13.6 − 3.6 = 10.0%).

Reproduction

In contrast to the effects of fish kairomones on the rate of reproduction and life history parameters, the effect of crowding information was negligible and significant only for size at first reproduction (CI in Table 1). This was because of a very minor and non-significant difference between the treatments without and with crowding information (nCI–CI in Table 2). General ANOVA for the effect of fish kairomones on reproduction for the pooled data from these two treatments revealed that in the presence of kairomones, maturation was accelerated (lower AFR), body size at first reproduction reduced (smaller SFR), number of eggs per clutch reduced, and per-offspring investment diminished (smaller egg volume). All of these changes were very apparent in each of the three replicate experiments (at least at $P < 0.05$) and in the entire data set (FK in Table 1). The effect of kairomones on each of the measured life history parameters (FK × CI in Table 1) was magnified by crowding chemicals, indicating a strong interaction. The % reduction of each parameter caused by kairomones (nFK–FK) was greater in the presence (CI) than in the absence (nCI) of crowding information (Table 2).

The effect of kairomones on reproduction was more apparent when clutch size was related to *Daphnia* body

Table 2 Two-way ANOVA for the effect of crowding information as a threat of starvation (CI) and the effect of fish kairomones as a threat of predation (FK) on the individual growth (growth rate), age (AFR) and size (SFR) at first reproduction, per-offspring investment (egg volume), number of eggs per clutch (clutch size), daytime depth selection (depth selected) and daily reproductive investment of *Daphnia* in the pooled data from all three experiments, given as the percentage difference (% diff) between the mean estimates (from all individuals in each treatment) in two-factorial experiments in the absence (nCI or nFK) or the presence (CI or FK) of information on another threat This means that nFK–FK is the difference (in % of the higher value) between the treatments without and with fish kairomones, either in the absence (nCI) or in the presence (CI) of crowding information, while nCI–CI is the difference (in % of the higher value) between the treatments without and with crowding information, either in the absence (nFK) or in the presence (FK) of fish kairomones. The significance of each difference for each trait from two-way ANOVA with post hoc Tukey's test is shown as *, ** and *** for $P < 0.05$, $P < 0.005$ and $P < 0.0005$, respectively

Diff	Treat	Growth rate % diff P	AFR % diff P	SFR % diff P	Egg volume % diff P	Clutch volume % diff P	Daily reproductive investment % diff P	Depth selected % diff P
nFK–FK	nCI	−13.6*	9.7***	13.0**	15.2*	−28.0*	42.0***	18.4*
	CI	−28.2***	17.4***	15.7***	31.2***	−31.5*	51.6***	44.1***
nCI–CI	nFK	−3.6ns	−3.8ns	−2.8*	−8.5ns	−7.2ns	8.2ns	2.2ns
	FK	14.0*	−5.0ns	−0.3ns	−11.9ns	−11.7ns	22.4ns	32.9***

size (Fig. 3a,b, Table 3), which sheds light on how the life history parameters were related to each other. The effect of kairomones on clutch size was negligible in spite of the major shift in age (AFR) and size (SFR) at first reproduction. The ANCOVA testing the difference in elevation of the regression lines indicated that crowding information caused a decline in clutch size and daily reproductive investment in the presence and absence of kairomones (Fig. 3). Furthermore, in both the absence and presence of crowding chemicals, the number of eggs per clutch was greater in the presence of kairomones than in their absence (Table 3), possibly due to smaller egg size, as shown by the two-way ANOVA (egg volume in Table 1), further confirming the well-known effect of fish kairomones on *Daphnia* and their use of life history adjustments as an antipredation defence.

Crowding information also caused a reduction in the number of eggs per clutch (to the same extent in the absence and in the presence of fish kairomones), and a decline in daily reproductive investment (to a much higher extent in the presence of kairomones, nCI–CI in Table 3, Fig. 3). This resulted in a larger egg volume in the absence of kairomones (nCI–CI, nFK in Table 2) and a smaller egg volume in the presence of kairomones (nCI–CI, FK in Table 2). Neither of these two effects was significant, but their interaction was significant (egg volume for FK × CI in Table 1).

Depth selection

In preliminary studies, no difference in daytime depth in the presence or absence of information on high population density was ever detected in the absence of fish kairomones. Nor was a difference observed in the single two-

factorial experiment among the 13 depth-selection experiments conducted in this study, although the negative depressing effect of kairomones in the presence of crowding chemicals was as strong as expected from earlier kairomone studies (Fig. 2c, Table 2).

In spite of high variability in the results, the daytime depth was found to be greater in the presence than in the absence of crowding information tested in the presence of fish kairomones (in 6 of 13 experiments), particularly when this information was from densities of 60 or more *Daphnia* L^{-1} and 4- to 7-day-old *Daphnia* were the test subjects (in 7 of 10 experiments, Table 4, Fig. 4). The depth difference increased with the intensity of the crowding information (Fig. 4b), with the slope of regression being non-significant for the lowest concentrations of this information (from 30 *Daphnia* L^{-1}), significant for six of eight experiments with information from 30 *Daphnia* L^{-1} and significant for all experiments with the two highest concentrations (90 and 120 ind. L^{-1}) at $P < 0.0004$ ($F > 16.5$, ANCOVA).

It was notable that the density required for the difference in depth selection to become evident was higher for younger and smaller *Daphnia* than for older and larger individuals. In the depth-selection experiments performed with the three density treatments of 30, 60 and >90 *Daphnia* L^{-1}, this difference was significant in 33, 43 and 100% of experiments, respectively.

The depth selected in the presence of crowding information was often twice that selected in its absence. This difference vanished in the dark (Fig. 4a) and was not apparent for the earliest instars (i.e. individuals younger than 3 days; 72 h in Fig. 4b). When considering the entire data set (Table 4), this difference was also evident in the slope of the regression of the difference versus individual
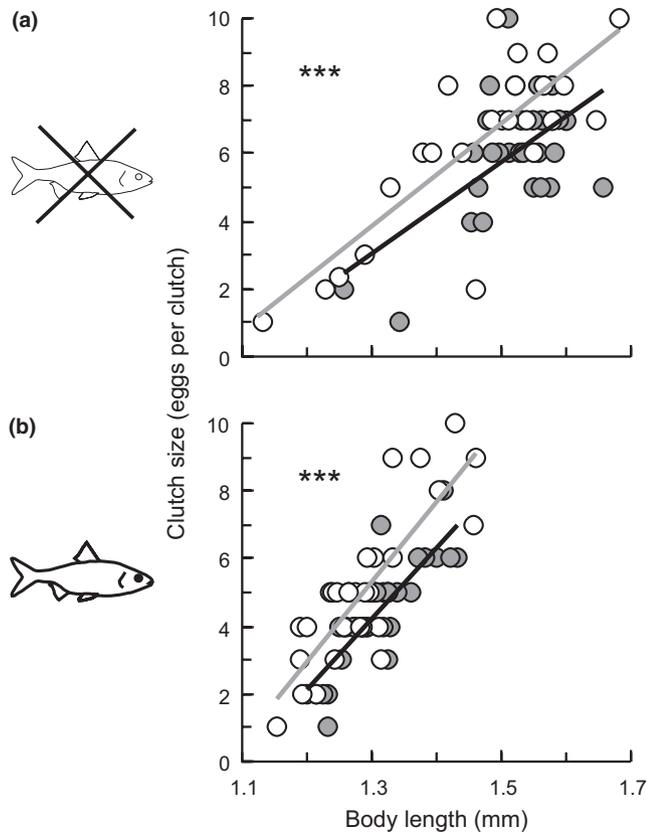
(a)

(b)

Fig. 3 Clutch size versus body length in 6- to 8-day-old 4th or 5th instar *Daphnia* grown individually in the absence (empty circles) or presence (filled circles) of crowding information from a *Daphnia* density of 60 ind. L^{-1}, in the absence (a) or in the presence (b) of fish kairomones (pooled data from experiments G1-3, statistics in Table 3, *** denote difference significant at $P < 0.0005$). The elevations of regressions from the two treatments in the presence of crowding information (black lines) do not differ at $P < 0.05$ (Table 3), but the clutch size was significantly greater in the absence than presence of kairomones ($P < 0.0001$, $F = 37.4$, ANCOVA). This was also found for daily reproductive investment (nFK–FK for *nCI* and *CI* in Table 2), the data for which showed a similar distribution to those for clutch size.

age, which was significantly different from zero ($P = 0.005$, $F = 7.68$, ANCOVA).

The single experiment (D13) examining a gradient of crowding information (0, 30, 60 and 120 *Daphnia* L^{-1}) revealed no effect of crowding chemicals in the absence of fish kairomones (Fig. 5a): the slopes of neither the linear nor the log-transformed regression lines were different from 0 at $P = 0.05$ (one-way ANOVA). However, the effect of crowding information was strong in the presence of kairomones, as revealed by the data integrated from the 13 experiments in Table 4 (Fig. 5b). There was a sharp increase in the difference in daytime depth in the absence and presence of crowding information during the initial phase of density increase from 0 up to 90 ind. L^{-1}. The

Table 3 ANCOVA statistics for the effect of crowding information as a threat of starvation (CI) and the effect of fish kairomones as a threat of predation (FK) on the number of eggs per clutch (clutch size) and daily reproductive investment (mm³ 10^{-3} day^{-1}) of *Daphnia* in the pooled data from all three experiments (G1–3), given as the difference (Diff) between the mean (from all individuals in each treatment) estimates in two-factorial experiments in the absence (nCI or nFK) or in the presence (CI or FK) of information on another threat (as in Table 2, data on clutch size related to body length are presented in Fig. 3). The significance of the difference between the elevation of each regression (Fig. 3) for the pooled data is shown as *, ** and *** for $P < 0.05$, $P < 0.005$ and $P < 0.0005$, respectively

Exp Diff	Treat	n_{ind}	Clutch size (egg per clutch) F and P	Daily reproductive investment (mm³ 10^{-3} day^{-1}) F and P
nFK–FK	nCI	56	12.85**	7.36*
	CI	59	11.70**	0.26[ns]
nCI–CI	nFK	52	12.63***	7.56**
	FK	63	15.25***	17.56***

Table 4 The effect of crowding information on the daytime (and the night-time in D9) depth selection by *Daphnia* in each of the 13 experiments (Exp D1–D13), given as the difference (Diff) between the mean (from n_{ind} individuals, each assessed 10 times a day) daytime depth selected in the presence (filled circles in Fig. 4) or in the absence (empty circles in Fig. 4) of this information for densities (ind. L^{-1}) of 30, 60, 90 and 120 *Daphnia* L^{-1} of 1- to 2-day-old (18–54 h) and 3- to 7-day-old (66–150 h) *Daphnia*, all in the presence of fish kairomones, except D13 (a two-factorial experiment). The significance of the difference is shown for each experiment as P at a given value of F (repeated measures ANOVA); *, ** and *** denote difference significant at $P < 0.05$, $P < 0.005$ and $P < 0.0005$, respectively

Exp	ind. L^{-1}	n_{ind}	40–150 h Diff	F and P	90–150 h Diff	F and P
D1	30	7	−0.01	5.43[ns]	−0.02	5.43[ns]
D2	30	6	−0.04	7.19*	−0.04	12.32**
D3	30	6	−0.01	0.94[ns]	−0.01	0.08[ns]
D4	60	10	−0.00	0.01[ns]	−0.02	0.50[ns]
D5	60	10	−0.02	0.84[ns]	−0.02	0.25[ns]
D6	60	10	−0.05	11.94**	−0.07	19.15***
D7	60	10	−0.04	2.96[ns]	−0.07	6.37*
D8	60	7	−0.04	3.24[ns]	−0.05	5.77*
D9	60	6	−0.10	48.68***	−0.11	51.66***
D9*	60	6	−0.01	0.02[ns]	−0.01	0.02[ns]
D10	60	6	−0.02	0.32[ns]	−0.03	1.67[ns]
D11	90	6	−0.11	23.70***	−0.12	27.06***
D12	120	7	−0.08	9.15*	−0.10	10.17**
D13[†]	120	6	−0.08	7.35*	−0.08	8.84*

*Night-time data.

[†]A two-factorial experiment that was performed at the beginning of this study whose results suggested the abandonment of treatments without fish kairomones. This suggestion also emerged from other two-factorial experiments performed with other *Daphnia* species (unpublished data).
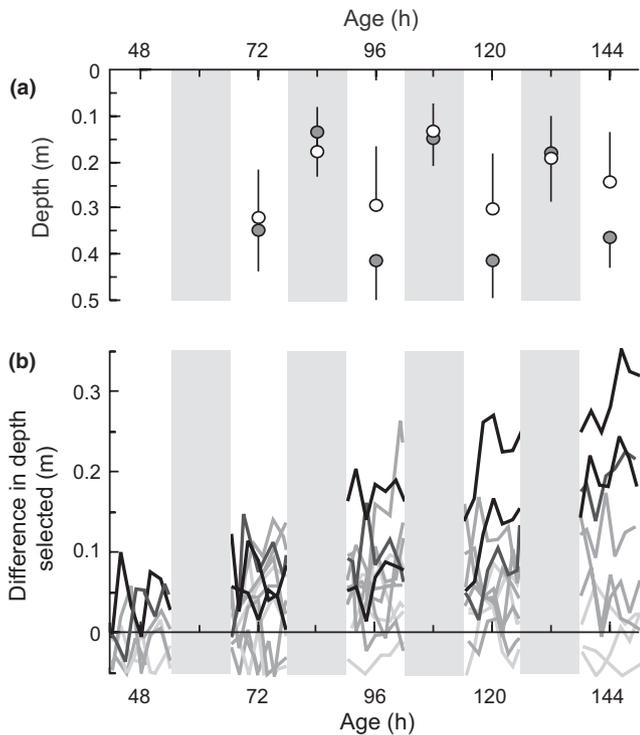
Fig. 4 Depth selection of individual *Daphnia* in the presence of fish kairomones, in the absence or presence of crowding information throughout their growth in the flow-through system, from 2-day-old juveniles (48 h) to 6-day-old females (144 h): (a) an example of mean (±1 SD) daytime (unshaded) and night-time (shaded background) depth selected by individual *Daphnia* in the absence (empty circles) or presence (filled circles) of crowding information from a *Daphnia* density of 120 ind. L^{-1} (six individuals × 4 days × 7 daytime + four night-time assessments per day in each of the two treatments, the difference significant at $P < 0.0005$, experiment D9, statistics in Table 4); (b) ontogenetic evolution of the difference in daytime depth between treatments without or with crowding information on population densities of 30, 60, 90 and 120 ind. L^{-1} (respectively, from light- to dark-shaded lines, each depicting daytime change in mean difference in depth selected by six individuals in experiments D1–D13, statistics in Table 4).

slope of the regression was different from zero ($F = 3.45$, $P < 0.0369$, ANCOVA).

Vulnerability to fish predation

All three experiments in which 300 *D. hyalina* were subjected to 30 min of predation by roach revealed a highly reproducible difference in the proportion of *Daphnia* consumed between those grown in the presence and in the absence of information on high population density, when they were offered to fish at the same density level. In spite of the variability in the outcome of the three experiments (Fig. 6), the percentages of *Daphnia* eliminated when grown in the presence and in the absence of information on high density were significantly different in
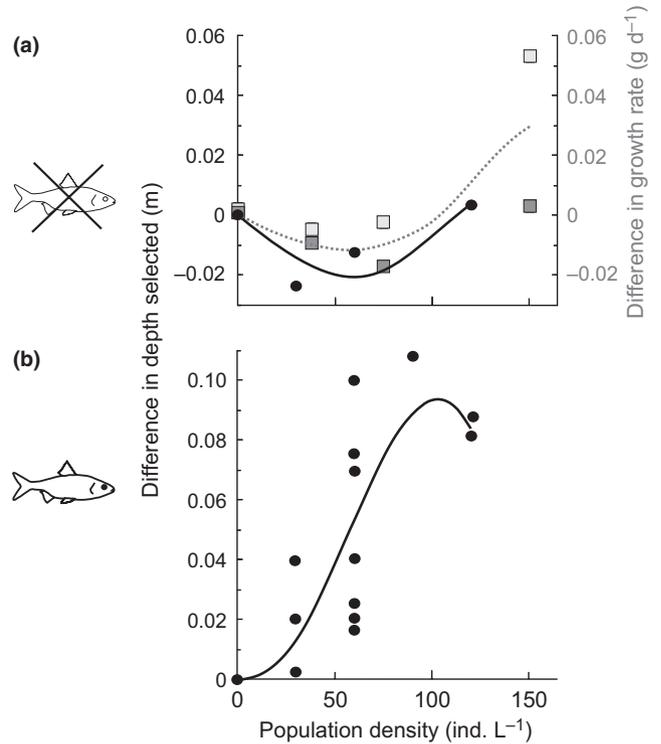
Fig. 5 The difference in daytime depth selected by individual *Daphnia* in the absence or presence of crowding information from *Daphnia* densities of 30, 60, 90 and 120 ind. L^{-1}, each in the absence (a) or presence (b) of fish kairomones (filled circles and black lines) from all 13 experiments listed in Table 4. Data extracted from fig. 2 in Burns (1995) on the difference in birth rate in *Daphnia* in the absence or presence of crowding information from different densities and in the absence of kairomones were added to panel (a) as squares for *Daphnia hyalina* and *Daphnia galeata* (dark and light shaded, respectively). Points represent observations; lines are the best fits selected by Excel Plot as multinominal type curves.

each case (24 and 53%, 49 and 63%, and 20 and 52% in the 1st, 2nd and 3rd experiments, respectively). The differences seen in the three experiments (14, 29 and 32%, respectively) were all significant at $P \leq 0.05$ according to the Kruskal–Wallis test, but slightly outside the margins of significance using Wilcoxon's test ($P = 0.08$). The number of survivors was always more than 1.5-fold greater in *Daphnia* grown in the presence of information on high population density.

Discussion

A rift between crowding and kairomone studies

Since the earliest studies on *Daphnia* demography, competition, life history and behaviour, examinations of the effects of high-density chemical information and of fish kairomones have often been completely separated from each other. This is in spite of the fact that each is equally
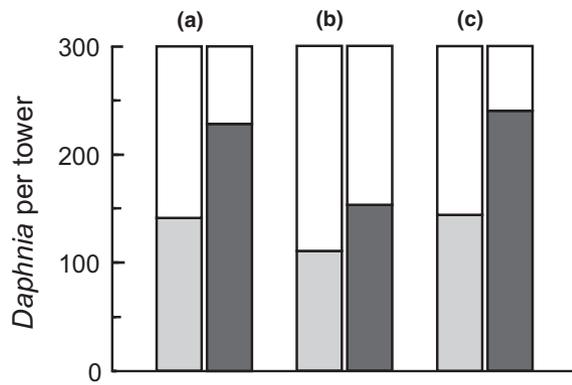
Fig. 6 Vulnerability to fish predation of 5-day-old *Daphnia* grown to the 4th instar in the absence (light shaded) or presence (dark shaded) of information on high population density levels. This vulnerability was assessed in each of three replicate experiments in February (a), April (b) and May (c) 2009 as the number of individuals surviving 30 min of predation by roach. Both towers were inoculated with 300 *Daphnia*, 15 min before the fish (two 2-year-old roach) were added, to produce an initial density of 1.5 ind. L^{-1}.

important to both components of *Daphnia* fitness: reproduction and mortality.

Since the initial studies of Frank (1952, 1957), investigations of the effects of high population density have remained focussed on food limitation, resource competition and declines in the rates of ingestion, individual body growth, fecundity and population increase (Ban *et al.*, 2008 and many other papers cited there), while ignoring the likely effect on *Daphnia* vulnerability to predation that may change with population density. The authors' reasoning has been dominated by the density-dependent effects of resource competition (Fig. 1a), with little attention paid to density-dependent predation risk (Fig. 1b). Moreover, they have generally examined the effects of crowding information from *Daphnia* cultures at densities exceeding the half-saturation density levels (½K in Fig. 1) that are typical of fishless habitats, rather than those with fish where predation does not permit high densities to persist except for the short period of the spring clear water phase (Lampert, 1988; Gliwicz & Wrzosek, 2008).

On the other hand, studies on the effects of kairomones, since those of Dodson (1989), Machaček (1991), Stibor (1992), Pijanowska, Weider & Lampert (1993) and Pijanowska (1994), have concentrated on predation risk, death rates and antipredation morphological, life history and behavioural defences, while ignoring the likely effects of crowding information on feeding, growth and reproduction, as is evident from the review by Tollrian & Harvell (1999). Their approach, focussed on predation risk and antipredation defences, has often ignored the density-dependent effects of resource competition (Fig. 1a). These

authors appear to have been reluctant to check whether the costly defences are displayed at low population densities, with most experiments being performed at high densities, most likely above half-saturation density levels (½K in Fig. 1) where it was possible to show that a *Daphnia* or *Bosmina* aggregation offers safety in the swarm, albeit in exchange for the costs of reduced feeding and growth owing to low food levels (Jakobsen & Johnsen, 1987, 1988; Pijanowska, 1994). This approach exploited the importance of a 'high-density antipredation refuge' that may occur when density increases beyond the point of half-saturation (½K in Fig. 1b) and ignored the possibility of an antipredation refuge produced by extremely low densities, when individual risk becomes reduced nearly to zero, a situation that is often observed in lakes with fish as 'summer *Daphnia* declines' (Sommer *et al.*, 1986).

It seems that recent reports have not contributed significantly to closing this rift, even though the possible interaction between the effects of information on increased risk and the effects of food stress (that may result from crowding) has been previously suggested (Pauwels, Stoks & De Meester, 2010).

Dual nature of crowding information in the presence of predation threat

The results of our two-factorial experiment did not confirm the notion that information on high *Daphnia* density should instigate declines in the rate of individual body growth. However, significant effects of crowding information alone were detected in some of the life history parameters (SFR) and the rate of reproduction represented as either clutch size or daily reproductive investment, substantiating the common belief that crowding chemicals carry information on impending food stress and the need to increase per-offspring investment (Glazier, 1992; Burns, 1995). These effects were not detected in depth selection. The absence of a major effect on growth rate could stem from the much lower *Daphnia* densities used in our study than those employed by Seitz (1984), Lee & Ban (1999), Burns (1995, 2000) and Rose *et al.* (2002). The density of 60 *Daphnia* L^{-1} used in our growth experiments was most likely below the half-saturation density level and certainly far below the carrying capacity (½K and K in Fig. 1) of *D. hyalina*, estimated to be 100–200 ind. L^{-1} in highly productive systems (Gliwicz, 2003; Gliwicz & Wrzosek, 2008), and representing the range of densities commonly used in earlier studies of crowding effects.

The effects of fish kairomones in our study were found to be as strong as in previous kairomone studies (see review by Tollrian & Harvell, 1999). They were stronger

than the effects of crowding information and apparent in growth rate, AFR, SFR, egg volume, clutch size and also depth selection. The effects of kairomones were opposite to those of crowding information in SFR, clutch size and daily reproductive investment: in their presence, *Daphnia* selected greater depths, their growth rates were reduced, and reproduction occurred earlier at smaller body sizes and produced a greater number of eggs of smaller size.

Our study revealed a strong interaction between the effects of kairomones and of crowding information that was most apparent when examined as the difference in the effect of kairomones in the absence and presence of crowding chemicals. This interaction stemmed from the additive effects of kairomones and crowding information on growth rates and depth selection, and from their opposing effects on SFR, clutch size, egg volume and daily reproductive investment.

The effect of kairomones on egg volume was much stronger in the presence than in the absence of crowding information that may signal impending food shortage, because *Daphnia* was either (i) unable to increase per-offspring investment because of its smaller body size and brood cavity at earlier first reproduction or (ii) reluctant to produce larger offspring that would be more vulnerable to visual predation.

This interaction was most spectacular in depth selection: the daytime depth selected by *Daphnia* in the presence of kairomones was much greater in the presence than the absence of crowding chemicals. The % difference between the depths selected in the absence and presence of kairomones was twofold greater in the presence of crowding information and was further increased with the increasing body size of subsequent instars, particularly when the intensity of the crowding information was greater.

These observations suggest that crowding information, apart from representing a cue indicating impending food stress and the need to increase per-offspring investment, carries a signal of increased risk of predation and the need to invest more in antipredation. The effect of this signal is most apparent in depth selection, one of the most flexible and most rapid antipredation defences, a behavioural adjustment to predation risk that may be less costly and swifter than alternate life history antipredation strategies such as diapause or other changes induced in life history traits (Tollrian & Harvell, 1999).

Two explanations why the response to fish kairomones is stronger at higher density level

The first possible explanation for the stronger response of *Daphnia* at high-density levels to fish kairomones may be adopted from the reasoning given by Pauwels *et al.* (2010), who suggested that under food stress (which is often a consequence of high population density), investments in growth, reproduction and further population increase are no longer adaptive, so *Daphnia* should reallocate resources and invest more in survival (i.e. replace a strategy to maximise reproduction with one to minimise mortality rate). Such reasoning could also be applied to explain the findings of the present study. When information on crowding indicates approaching food stress, the presence of fish kairomones should cause *Daphnia* to reduce their investment in growth and reproduction in an attempt to strengthen their antipredation chances and remain alive for as long as possible, until food availability and safety improve, perhaps also by selecting greater depths where lower temperature should permit increased longevity, as suggested by Dawidowicz & Loose (1992) and De Meester *et al.* (1999).

The second possible explanation arises from the notion of 'low-density antipredation refuge' (Fig. 1b). Below a threshold population density, optimally foraging harvesters such as planktivorous fish either switch to alternate prey categories or migrate away in search of more profitable locations with higher densities of prey (Gliwicz & Wrzosek, 2008). Therefore, at low population densities, *Daphnia* should suppress costly antipredation defences and reallocate resources to growth and reproduction. This reasoning could also provide another possible explanation for the findings of Pauwels *et al.* (2010). When information on high food abundance indicates low population density, the presence of fish kairomones as a warning of increased risk should be ignored by *Daphnia*, and resources reallocated into increased growth and reproduction or into the production of diapausing eggs when forthcoming starvation is expected (Ferrari & Hebert, 1982; Slusarczyk, 2001; Fitzsimmons & Innes, 2006).

While the second explanation reflects the role of information on high population density in reinforcing antipredation in the presence of kairomones, the first, following the arguments of Pauwels *et al.* (2010), takes account of the effects of kairomones in the reaction to anticipated food stress (resulting from crowding). In spite of their different approaches, the present study and that of Pauwels *et al.* (2010) have a similar message: that the defensive reaction of *Daphnia* to fish kairomones is stronger under high than low population density conditions (and at low rather than high food levels). However, it is not clear which of the two possible scenarios (high density as a signal of impending food stress or increased predation risk) is more likely and whether the two are mutually exclusive or additive to each other.

In interpreting our findings, the notion of 'low-density refuge' seemed to be an equally attractive alternative. Discarding defences allows more energy to be allocated to growth and reproduction at low population densities when predators remain attracted to alternate prey categories or to more prey-proficient sites. This view is supported by the fact that most of the effects of crowding information, at the intensities used in our study, were too small to be detected in the absence of kairomones but very apparent in their presence (Table 1); the difference between the treatments without and with fish kairomones in the presence of crowding information was twice that in its absence. This notion was further supported by the results of the fish predation trials, which revealed that acclimation of *Daphnia* to the risk of predation when grown in the presence of fish kairomones allowed them to respond more rapidly to imposed predation with an enhanced ability to evade the experimental fish. This could stem from their higher sensitivity to light and their more rapid evasion by descent to greater depth in 'plankton towers'. This possibly represents the quickest behavioural defence that does not require any experience from earlier encounters with the predator.

However, there are three reasons for a general reluctance to accept the 'low-density-refuge' explanation. The first lies in the different ranges within the population density spectrum that represent each of the two approaches; the densities of the maximum individual risk that are right between the two density ranges (Fig. 1b) are often regarded as a low-density situation when compared to that within a dense aggregation. The second lies in the different ways of looking at the inducible defences of zooplankton, focussing on either the ability to switch them on, as in the reviews of Dodson (1989) and Tollrian & Harvell (1999), or the ability to suppress them when they are not needed, in an effort to reallocate resources to growth and reproduction, as shown in this study. The third lies in the different ways of looking at a *Daphnia* aggregation: either as a refuge from fish predation (Milinski & Heller,1978; Jakobsen & Johnsen, 1988; Pijanowska, 1994) or as a site of highly efficient feeding for planktivorous fish exploiting patches of prey (McNaught & Hasler, 1961; Jackson *et al.*, 2005; Ioannou *et al.*, 2009).

The two explanations of enhanced *Daphnia* growth at low population density do not have to be considered as strict alternatives. Whatever the subject of the 'forewarning' conveyed by high population density (impending starvation or increased predation), the need to invest in survival goes side by side with the need to invest in defences, the latter being a component of the former. It is possible that crowding information carries not only a cue indicating approaching food stress (as known from earlier studies), but also signals the need to activate defences. Activating antipredation defences at high density may reflect a life history shift from maximising birth rate (non-adaptive in cases of approaching food shortage) to minimising death rate (as suggested by Pauwels *et al.*, 2010). Suppressing defences at low-density levels may reflect a shift from investing in behavioural and life history defences to investment in faster growth and increased reproduction, while taking advantage of a time window when the 'low-density antipredation refuge' can be exploited (as suggested in this study). The phenomenon of discarding defences in the absence of crowding information seems highly adaptive, regardless of whether it is a response to the perception of approaching food abundance or the reduced risk of predation, or both.

Acknowledgments

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