Threat or treat: the role of fish exudates in the growth and life history of Daphnia

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Abstract. In kairomone studies, it is often implicitly assumed that the only effect of predator exudates on their prey is to trigger anti-predator defenses. However, chemicals originating from fish activity may also fertilize the environment and enhance the growth of zooplankton prey by increasing bacterial food availability. It is necessary to separate these two effects in order to examine the adaptive significance of zooplankton anti-predation defenses and the ability of the prey to benefit from fish-related food.

Here, we have employed differential filtration of media to permit assessment of these two effects on growth rate and life history adjustments in two Daphnia species that differ in body size and the ability to collect small particles (greater in small-bodied D. cucullata than in larger D. hyalina). Filters of three mesh sizes were employed: 2 μm (most bacteria pass, but detritus is retained), 0.45 μm (standard) and 0.20 μm (most bacteria and detritus retained).

The concentration of kairomones in the fish medium was assumed to be unaffected by filtration. However, the abundance of bacteria in the fish and no-fish media was dependent on the filter mesh size. This influenced the food level and its impact on Daphnia growth rate and life history. The effect of fish-related bacterial food was greater under low than under high algal food conditions (0.1 and 1.0 mg C/L) and more significant in D. cucullata.

Use of media with a diminished bacterial abundance revealed the effect of kairomones alone as (1) reduced growth rate, size at reproduction, number of pre-adult instars, age at maturation, level of the integrated egg and body lipids, egg volume, and (2) increased number of eggs per clutch vs. body length. These effects were equally strong in both species, despite differences in body size, and were greater at the higher algal food concentration. The findings of this study indicate the need to re-analyze and possibly revise the results of previous kairomone studies, especially those using media filtered through mesh sizes coarser than 0.2 μm that were performed under limiting algal food conditions or with species particularly able to benefit from bacterial food.

Key words: bacterial food; filter mesh size; food limitation; growth rate; kairomones; life history.

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INTRODUCTION

The common occurrence of chemical communication in aquatic ecosystems stems from the persistence of infochemical gradients in media of high viscosity, such as water, when compared with the turbulent atmosphere (Pohnert et al. 2007). In lake water, where the reliability of
visual cues can be obscured either by high turbidity or low ambient light intensities (Vinyard and O’Brien 1976), chemical communication is a suitable and effective way of acquiring information about the environment (Havel 1987, Tollrian and Harvell 1999). Among known infochemicals, kairomones have received much attention over the last 25 years as a result of studies on inducible reactions in animals. Kairomones are semiochemicals (chemical signals that transmit information between individuals; Law and Regnier 1971) mediating interspecific interactions that benefit the receiver rather than the emitter (Brown et al. 1970). While the majority of studies on kairomones in terrestrial habitats concern substances used by foraging organisms to locate the emitter as a possible food source (‘foraging kairomones’, Kats and Dill 1998), in aquatic systems, the focus is more on kairomones as signals warning of the risk of predation (Larsson and Dodson 1993). The ability of a potential prey to detect such predator-related kairomones (Ruther et al. 2002) and phenotypically adjust aspects of its life history, morphology and behavior may result in better predator avoidance or predator resistance (Havel 1987, Larsson and Dodson 1993, Tollrian and Harvell 1999).

Predator-released compounds not only carry important information on the current risk of predation, they may also indirectly (e.g., nutrients released by herbivorous zooplankton for algae and bacteria) or directly (nutrients released by vertebrate and invertebrate predators for herbivores) represent a source of high-quality food that may enhance the growth and fecundity of the prey. Therefore, to appreciate the full ecological significance of kairomone-induced anti-predation defenses it is vital to separate the different effects of these factors on prey species. This is a challenging task while the chemical nature of kairomones remains unknown. To date, only a few infochemical cues have been characterized and can be applied to experimental systems as pure substances (Kusch and Heckmann 1992, Yasumoto et al. 2005). In many cases, difficulties have been encountered when attempts have been made to recognize the source and chemical (or biochemical) nature of kairomones (Burks and Lodge 2002, Pohnert et al. 2007). Therefore, rather than by adding the pure substance, most experimental work on kairomone-inducible defenses has been carried out using media based on filtered lake water in which the predator or injured conspecifics were once present (e.g., Machácek 1991, Stibor 1992, Stibor and Luning 1994). Although this approach has considerable limitations, it is now well established in ecological and evolutionary research and has been employed in numerous studies on prey-predator interactions in freshwaters, most examining the relationship between planktonic animals and planktivorous fish (Larsson and Dodson 1993, Lass and Spaak 2003).

It is clear that some bacteria remain even in thoroughly filtered lake water. Moreover, the bacterial biomass in experimental media increases over the course of an experiment and bacterial growth is stimulated by the increased nutrient concentrations resulting from the presence of a predator or injured prey before media preparation. For this reason, accurate determination of the overall effect of kairomones and alarm substances is difficult (easily under- or overestimated) even under ‘controlled’ laboratory conditions. The presence of readily digestible surplus predator-related food can lead to significant underestimation of the growth reducing force of kairomones in otherwise well constrained planktonic herbivore life history experiments. Overestimation of the effects of kairomones may be observed in studies on the depth selection of Artemia and Daphnia (Forward and Rittschof 1999, Ringelberg and Van Gool 1998, respectively), where animals occupy the deeper layers of the water column in the presence of fish exudates and feces, partly due to the decreased threat of predation (Dodson 1988), but possibly also because relatively higher concentrations of bacterial food are present in the deeper strata.

Overestimation of the effect of kairomones may also result from the stronger reaction to these infochemicals at higher food levels (Lima and Dill 1990). Since satiated animals are able to expend more energy on risk avoidance, they are more likely to accept later maturation in the deep, dark and cold hypolimnion (Hays et al. 2001), or a slower growth rate due to life history adjustments (Gliwicz and Maszczyk 2007). A good example of possible exaggeration of the effect of kairomones is provided by the ciliate Euplotes octocarinatus, which exhibits morpholog-
ical defences in response to predator cues released by another ciliate *Stylonychia mytilus*, but only at high food levels (Wiackowski and Szkarlat 1996). Such overestimation might also have been observed in studies on the freshwater bacterium *Sphingobium* that forms aggregates when exposed to predation by a protistan (*Poterioochromonas* sp.), a response that is probably not only due to the presence of specific chemical cues (Pernthaler et al. 1997, Blom et al. 2010), but may likewise be caused by surplus substrates and nutrients released by the predator into the surrounding medium (Blom et al. 2010). This may also apply to algae that form grazing-resistant colonies in the presence of the herbivorous *Daphnia* and rotifers (Hessen and Van Donk 1993). The overestimation of the effect of kairomones might be attributed to a variety of other behavioral and phenotypic responses to ‘predator medium’, such as horizontal migration, and spine or helmet induction in rotifers, ciliates and cladocerans (Gilbert 1966, Kuhlmann et al. 1999, Burks and Lodge 2002, respectively).

The need to distinguish the effect of the threat of predation from that of a predator-related food ‘treat’ seems to be of high importance in order to appreciate the adaptive significance of the ability of the prey to benefit from predator-related food. Many small-bodied zooplankton species co-exist successfully with planktivorous fish due to the relatively minor impact of this predator on their community structure and population density (Vanni 1987). Therefore, it is expected that small-bodied zooplankton species may invest less energy in predator avoidance or resistance strategies, either by (1) exhibiting a weaker reaction to the presence of kairomones (Dodson 1988), or (2) choosing less costly defense strategies (e.g., life history induction in rotifers rather than depth selection adjustments, Leibold and Tessier 1991). Since smaller-bodied species (or clones) would more commonly coexist with planktivorous fish and have greater food requirements (Gliwicz 1990), they are possibly better adapted to exploit and benefit from the presence of fish-related bacterial food. Until recently, these bacteria have been associated only with the production (Ringelberg and Van Gool 1998, Beklioglu et al. 2006a) or decomposition of kairomones (Loose et al. 1993, Beklioglu et al. 2006b, Akkas et al. 2010), and have not generally been considered as an additional food source for planktonic animals. To our knowledge, only one study (Vijverberg and Vos 2006) has demonstrated that the presence of fish can affect the feeding conditions of planktonic animals. However, this was interpreted as a result of an improvement in food quality rather than its higher quantity.

The aim of this study was to try and disentangle the signaling effect of fish kairomones from the nutritional effect of fish-related supplementary bacterial food (at low and high algal food concentration) on the growth rate and plasticity of the life history traits of two *Daphnia* species (*D. cucullata* and *D. hyalina*) common in European lakes. Such an experimental approach allowed us to test (1) whether fish exudates and feces can affect the growth, reproduction and survival of zooplankton, not exclusively due to the presence of kairomones, but also due to the input of fish-related bacteria, (2) if the effect of surplus microbial food related to the presence of fish is greater at low than at high algal food concentration and is more important in the smaller species that is less vulnerable to fish predation, and (3) whether the effect of kairomones alone (solely as information on predation risk) is greater in the larger bodied species, and at high rather than low algal food concentration.

**Material and Methods**

**The approach**

Each of the three experiments was performed using two *Daphnia* species and a 2 × 2 factorial design including fish-conditioned and non-conditioned (control) water medium, at high and low algal food level (*Scenedesmus obliquus*) at initial concentrations of 1.0 and 0.1 mg C/L, respectively, with food concentrations ranging from 96 to 78% of the starting values at the time of media replacement. According to preliminary experiments, it was assumed that the concentration of fish kairomones in fish treatments was the same in each experiment (the effect of kairomone degradation by bacteria was negligible). To test the effect of additional bacterial food on the growth rate and life histories of *Daphnia*, the abundance of bacteria in each experiment (high, medium and low; *E*<sub>high</sub>, *E*<sub>m</sub> and *E*<sub>low</sub>, respectively) was modified by employing different filtration protocols.
From the start of each experiment, animals were individually cultured in 100 ml beakers (containing 90 ml of medium) from the early neonate stage (0–6 h after birth) until the second adult instar. The culture beakers were placed in a water bath to ensure a constant temperature of 20 ± 0.1°C and illuminated with a summer photoperiod (16 h light:8 h dark). The lake water (eutrophic Lake Gora, Warsaw, Poland) used in all experiments was aged (>14 days), filtered (2.0 μm, Sartorius) and aerated to remove possible traces of information on conspecifics or predators (kairomones, Loose et al. 2006). Experimental media were prepared and replaced every 16 hours. In this study, a filtration protocol instead of antibiotic treatment (which has been occasionally recommended, Ringelberg and Van Gool (1998, Beklioglu et al. 2006) was used to remove bacteria (Ozaktas 2007); second, we were aware of possible side effects of these compounds on the experimental animals that could potentially affect their life history features; and third, we aimed to obtain results comparable with those of earlier kairomone studies and therefore we employed a standard filtration method (Macháček 1991, Stibor 1992, Doksaeter and Vijverberg 2001).

Prior to filtration, half of the lake water (25L) was conditioned by adding five small planktivorous fish (10 ± 1 cm, roach [Rutilus rutilus]), and holding the water at a constant temperature of 20 ± 0.1°C for 16 h. The roach were transferred from a separate tank where they had been fed once a day with a standard amount (~20 ind. per fish) of frozen Chironomidae larvae. The other half of the lake water was held at the same temperature in the same type of 30 L aquarium without fish (control).

**Experimental animals**

*Daphnia hyalina* (clone HG011, Lake Swiecajty, Poland) and *D. cucullata* (clone C006, Lake Tjeukemeer, Netherlands) used in the experiments are known to exhibit strong phenotypic responses to the presence of fish medium (Gliwicz et al. 2012). To minimize possible maternal effects (Lynch and Ennis 1983), all animals came from the second clutch of fourth-brood females from long established synchronized laboratory cultures that had been maintained in 4 L jars with aged (>14 days), aerated and 2.0-μm-filtered lake water, and fed daily with *Scenedesmus* at a concentration above the incipient food level (1.0 mg C/L).

**Experimental design**

At the beginning of each experiment, 320 synchronized neonates (160 per species) were randomly distributed between four treatments (80 individuals per treatment): fish-conditioned or control water medium, at low or high algal food level.

Depending on the experiment, both the control and kairomone media were filtered through 2.0, 0.45 or 0.20-μm Sartorius membrane filters (in E_high, E_m and E_low respectively). The filtration procedure used in E_high (2.0 μm) allowed the greatest number and biomass of bacteria to remain, but removed all suspended particles, while the procedures used in E_m (standard in kairomone studies) and E_low resulted in a step-wise decrease in the biomass of available bacterial food and minimized the difference in bacterial levels between the fish and control media. Filtered fish-conditioned and control water was then mixed with different volumes of algal suspension (initial concentrations) in which the organic carbon content had been calculated from a calibration curve relating organic carbon to absorbance at 800 nm.

To determine the initial size and the level of ovary lipid droplets in the *Daphnia*, 20 randomly chosen neonates from each species were photographed under a dissecting microscope. They were then preserved in formalin (25%), rinsed, dried (12 h at 60°C) and weighed on a microbalance scale (Orion-Cahn C-35). By examination of the photographs using MultiScan image analysis software (Computer Scanning Systems) each individual was measured from the top of the eye to the base of the tail spine and the amount of body lipid reserves was calculated as the cumulative volume of ellipsoidal droplets (droplet volume was calculated from the shortest and the longest diameters).

In each experiment, all animals were checked for molting and survival every 8 hours during the juvenile stage and the first two adult instars. Each successfully molted individual was photographed and their size and the volume of lipid
reserves were determined as described above. Simultaneously, every 24 hours, two randomly chosen animals from each treatment were taken for body mass estimation. To determine the somatic growth rate, 6 individuals at first reproduction (same physiological stage) from each species in each treatment were dried and weighed. Body growth rate was determined as \( g = \frac{\ln C_t - \ln C_0}{t} \), where \( C \) is the body mass of a newborn neonate \( (C_0) \) and an adult \( Daphnia \) after oviposition \( (C_t) \).

When the first and second clutch of eggs had been deposited into the brood chamber (first and second reproduction) the following life history parameters were recorded for each \( Daphnia \): age and size at reproduction, number of eggs per clutch and the volume of each egg (where visible) in the brood cavity (calculated as for lipid droplets). In addition, when the first eggs were laid, lipid droplets in the body were counted and taken to represent reserves remaining after reproduction. Total lipid volume was calculated as the sum of the integrated droplet volumes in eggs, plus the reserves remaining in the body.

**Nutritional currencies of the bacterial and algal food**

**Organically bound phosphorus content.**—The initial values (immediately after filtration) of organically bound phosphorus associated with bacteria \( (P_{orgB}) \) were determined in triplicate samples (1000 ml) of both the fish and control media taken during the first and the last days of each experiment (each time prior to mixing the media with the algal food). \( P_{orgB} \) was calculated as the difference between total phosphorus (TP) and the cumulative amount of reactive orthophosphates with acid-hydrolysable P. To determine the initial TP content of each sample, half of the volume was ashed at 550°C for 6 hours, suspended in 10 ml distilled water and 2.4 ml of 10 N \( \text{H}_2\text{SO}_4 \), and digested at 100°C to solubilize reactive orthophosphates. To determine the level of acid-hydrolysable P among the reactive orthophosphates, the other half of the sample volume was autoclaved at 120°C with \( K_2\text{S}_2\text{O}_7 \) for 2 hours to convert acid-hydrolysable P into soluble reactive orthophosphates. Finally, the concentration of soluble reactive orthophosphates in each half of the sample was determined using the standard stannous chloride method (Murphy and Riley 1962).

The initial content of organically bound P in algae \( (P_{orgSc}) \), at both \textit{Scenedesmus} concentrations \((1.0 \text{ and } 0.1 \text{ mg C/L})\), was determined in triplicate volumes (1000 ml) of sterilized (boiled) and filtered (through 0.2 \( \mu \text{m}-\text{filter mesh size to remove bacteria} \) water that had been mixed with algal food suspension during the first and the last days of each experiment. The analytical procedure was the same as that used for \( P_{orgB} \) determination.

**Particulate organic carbon content (POC).**—The particulate organic carbon content of bacteria and algae was calculated separately through enumeration of their cells. This was done on the first and last days of each experiment for triplicate 15-ml samples of each freshly prepared medium and subsequently for samples of each medium 8 and 16 hours after the filtration procedure (each medium was mixed before samples were taken). Each sub-sample of medium was fixed by adding formaldehyde solution (final conc. 2%), and analyzed within 14 days using epifluorescence microscopy.

After standard DAPI staining (Porter and Feig 1980), the bacterial cell number in each sample was counted in 14 random fields of view with a Nikon Eclipse E400 microscope equipped with an epifluorescence device using NIS Elements 2.3 software. The total number of bacteria (ml\(^{-1}\)), was calculated using the equation \( \text{NB} = n \times \text{FV/\( \text{Vfv} \times V \)} \), where \( n \) is the mean number of bacteria in a single field of view, FV/\( \text{Vfv} \) is the ratio between the total filter area \( (\text{FV} = 254,469 \, \mu \text{m}^2) \) and the cumulative area of 14 analyzed fields of view \( (\text{Vfv} = 5519 \, \mu \text{m}^2) \), and \( V \) is the sample volume. The particulate organic carbon content of bacteria \( (\text{POC}_{bac}) \) was calculated using the Psenner equation (Psenner 1993) based on bacterial cell counts.

Algal cells in each sample were subsequently counted in the same 14 fields of view, using red auto-fluorescence for identification. The particulate algal organic carbon in each sample was calculated from a calibration curve, relating the POC of \textit{Scenedesmus} to the number of cells, prepared before the start of the experiments.

**Efficiency of exploitation of bacterial food**

The inter-setular distances in the filtering screen of \textit{Daphnia} in each experiment were
measured by light microscopy (×1600 immersion lens) for three randomly chosen individuals from each experimental treatment on the 3rd and 8th–10th day of life, i.e., after the instar with a minimum volume of accumulated lipid reserves and during the second adult instar, respectively. Prior to measurement, the animals were preserved in 4% sugar formaldehyde and the endopodites were dissected from the third pair of thoracic legs of each individual (two endopodites per Daphnia) and transferred in a drop of glycerol onto a microscope slide (Bednarska and Dawidowicz 2007). The filter screens were then photographed and inter-setular distances were measured at 10 locations in the middle of the setae using MultiScan image analysis software.

**Results**

**Cumulative data from all experiments (E\textsubscript{high}, E\textsubscript{m}, and E\textsubscript{low}).**

Phosphorus content.—The cumulative data from all experiments revealed that the initial concentrations of organically bound P associated with bacteria (P\textsubscript{orgB}) as well as the percentage of the initial P\textsubscript{orgB} to the initial P\textsubscript{orgB+Sc} were higher in the fish medium than the control, and lower in media filtered through the smaller mesh size filters (p < 0.0020, ANOVA, Table 1).

Particulate organic carbon content (POC).—The initial and the mean (1) particulate organic carbon of bacteria (μPOC\textsubscript{B} and meanPOC\textsubscript{B}, respectively), (2) percentages of POC\textsubscript{B} to total POC of the algae and bacteria (μPOC\textsubscript{B} × μPOC\textsubscript{B+Sc} × meanPOC\textsubscript{B+Sc}⁻¹ and meanPOC\textsubscript{B} × meanPOC\textsubscript{B+Sc}⁻¹, respectively), as well as (3) the number of bacteria (μN\textsubscript{B} and meanN\textsubscript{B}, respectively) were (1) higher in fish medium than the control (p < 0.0001, Table 1), (2) lower in media filtered through smaller mesh size filters (p < 0.0001, Table 1), and (3) not influenced by the algal concentration (three-way ANOVA). The difference between the mean (±1 SD) POC of bacteria in fish medium and the control was highest in E\textsubscript{high} (0.26 ± 0.05 mg C/L), moderate in E\textsubscript{m} (0.09 ± 0.03 mg C/L) and lowest in E\textsubscript{low} (0.01 ± 0.00 mg C/L). The difference in mean POC of bacteria between the control and fish medium was insignificant only in E\textsubscript{low} (two-way ANOVA). POC of algae decreased slightly during the 16 h between filtration procedures (four-way ANOVA, p < 0.0411). The C:P ratio (cumulative data from each treatment in each experiment) was approximately ten times lower for bacteria than for algae (9.3 ± 7.7 and 104.1 ± 13.4, respectively).

The filtering apparatus.—The mean (±1 SD) inter-setular distances in the filtering screen of juvenile Daphnia cucullata (Dc) were smaller than those of juvenile D. hyalina (Dh) (0.31 ± 0.04 and 0.41 ± 0.03, respectively, p < 0.0001, rmANOVA) for each treatment in each experiment (n = 360, 10 inter-setular distances measured in each of 3 individuals). In adults (after the second adult instar) the difference was smaller (0.51 ± 0.04 in Dc, and 0.56 ± 0.05 in Dh, p < 0.0001, rmANOVA). In both juveniles and adults, elevation of the regression of inter-setular distance versus body length in Dc was lower (ANOVA, p < 0.0001), and the slope (ANOVA, p < 0.0012) higher, than in Dh.

The factors and interactions affecting Daphnia growth and life history parameters.—Bacterial food (B factor, different numbers of bacteria in subsequent experiments resulting from different filtration procedures during media preparation) affected the individual growth rate (GR), age and size at first (AFR and SFR, Table 2) and second reproduction (ASR and SSR, p < 0.0005, three-way ANOVA) in both Daphnia species. However, a higher level of this food reduced the number of pre-adult instars, and increased (1) egg volume, (2) the volume of body lipid reserves during the second instar, and (3) the integrated volume of lipid reserves (in body and eggs) in the first-clutch females, but only in Dc (Table 2). Furthermore, only in Dc, the decrease in the volume of natal lipid reserves during the first two instars was considerably slower at high (E\textsubscript{high}) than at low (E\textsubscript{low}) bacterial food level (comparison of slopes, ANCOVA, Table 3). There was 100% survival of Dh to the second reproduction in each experiment, but for Dc this occurred only in the experiment with the high bacterial level (E\textsubscript{high}). During the experiment with the intermediate bacterial level (E\textsubscript{m}), survival to second reproduction ranged from 85% (in the control at the low algal food concentration) to 97% (in the fish medium at the low algal food concentration), and all mortality occurred between days 2 and 3. The lowest rate of survival to second reproduction occurred at the low bacterial food level (E\textsubscript{low}, survival ranged from
Table 1. The initial quality of bacterial food shown as organically bound phosphorus ($\mu$P$_{org}$) and the percentage of $\mu$P$_{org}$ to total organically bound phosphorus ($\mu$P$_{org}$/Sc), the initial and mean quantities of bacterial food shown as the particulate organic carbon content ($\mu$POC and meanPOC, respectively), the percentage of POC$_B$ to total POC, and the number of bacteria ($N_b$) in the course of each of the three experiments (E$_{high}$, high; E$_{low}$, medium; and E$_{low}$, low bacterial food level; indicated in the second row of column headings). Each value is shown for high and low algal (Scenedesmus obliquus) food concentrations (indicated in the top row of column headings), in no-fish (control, C) and fish (F$_m$) medium. All POC estimations ($n = 252$), as well as all P$_{org}$ estimations ($n = 252$) are the means for 3 samples ($n = 3$) taken twice during each of the experiments (on the first and the last days, $n = 6$), while bacterial cell numbers were counted in 14 fields of vision ($n = 14$). POC estimations were made for both the initial and the mean value (0 h and 0, 8 and 16 hours after the filtration procedure, respectively). P$_{org}$ estimations were made only for the initial value (0 h after the filtration procedure).

<table>
<thead>
<tr>
<th>Trait</th>
<th>High</th>
<th>Medium</th>
<th>Low</th>
<th>Low</th>
<th>Low</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C</td>
<td>F$_m$</td>
<td>C</td>
<td>F$_m$</td>
<td>C</td>
</tr>
<tr>
<td>$\mu$P$_{org}$ (mg/L)</td>
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<td>9.2</td>
<td>0.6</td>
<td>1.1</td>
<td>0.2</td>
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<tr>
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<td>2</td>
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<td>2</td>
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<tr>
<td>$\mu$POC (mg/L)</td>
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<td>0.00</td>
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<tr>
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<td>10</td>
<td>8</td>
<td>19</td>
<td>1</td>
</tr>
<tr>
<td>meanPOC (mg/L)</td>
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<tr>
<td>meanPOC$_B$ (%)</td>
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<td>24</td>
<td>1</td>
<td>11</td>
<td>1</td>
</tr>
<tr>
<td>$\mu$N$_b$ (no. cells $\times 10^5$)</td>
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<td>14.7</td>
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<tr>
<td>mean$\mu$N$_b$ (no. cells $\times 10^5$)</td>
<td>8.0</td>
<td>29.0</td>
<td>1.0</td>
<td>8.1</td>
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Table 2. Analysis of data from all experiments (E$_{high}$, E$_m$ and E$_{low}$) by three-way ANOVA ($F$, $p$ and degrees of freedom) to test the significance of the effect of bacterial food (B), algal food (Sc), fish medium (F$_m$), and interactions between these factors in D. hyalina (Dh) and D. cucullata (Dc) on individual growth rate from newborn neonates to ovigerous adults (GR), size at first reproduction (SFR), age at first reproduction (AFR), the number of pre-adult instars (Instars), minimum volume of body lipid reserves (Lipid volume; Juvenile), integrated egg and body volume of lipids in the first-clutch females (Lipid volume; Adult), and egg volume. Statistical significance is accepted at *$p < 0.05$, **$p < 0.005$ or ***$p < 0.0005$. NS indicates no significance.

<table>
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<tr>
<th>Factor, interaction</th>
<th>GR</th>
<th>SFR</th>
<th>AFR</th>
<th>Instars</th>
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<th>Adult</th>
<th>Egg volume</th>
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<td>$p$</td>
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<tr>
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</tr>
<tr>
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<td>16</td>
<td>***</td>
<td>20</td>
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<td>***</td>
<td>118</td>
<td>***</td>
<td>32</td>
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<tr>
<td>B $\times$ Sc</td>
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<td>9</td>
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<td>5</td>
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<tr>
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<td>*</td>
<td>3</td>
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<td>0</td>
</tr>
<tr>
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<td>NS</td>
<td>4</td>
<td>NS</td>
<td>0</td>
<td>NS</td>
<td>0</td>
</tr>
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<tr>
<td>B</td>
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<td>*</td>
<td>4</td>
<td>*</td>
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<tr>
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<td>6</td>
<td>*</td>
<td>3</td>
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Notes: Error degrees of freedom for Dh: GR, 74; SFR, AFR, instars, 344; lipid volume for juveniles and adults, 304; egg volume, 319. Error degrees of freedom for Dc: GR, 72; SFR, AFR, instars, 304; lipid volume for juveniles and adults, 303; egg volume, 305.
50% in the control to 69% in the fish medium, at both algal food concentrations). Notably, between 74-100% of individuals died between the 2nd and 4th days of life.

In contrast to the effect of bacterial food, algal food (Sc factor, low or high concentration of algae) improved feeding conditions for Dh to a greater extent than for Dc (Table 2). Only in the case of the number of pre-adult instars was the influence of this effect reversed.

The presence of fish medium (Fm factor, kairomones and fish-related bacteria) generally reduced GR (Fig. 1), as well as body size in subsequent instars (Fig. 2), egg volume, SFR (Table 2), and SSR (p < 0.0001, three-way ANOVA) in both species. The exception was GR at the high bacterial food level (Ehigh) and body size (SFR, SSR) at high and medium bacterial food level (Ehigh and Em). The effect of the Fm factor was evident in the acceleration of AFR (Fig. 3) and ASR in both species (Table 2). In Dh, first reproduction was observed mostly in the 5th instar (100% of animals in the control and 90–97% in the fish medium), while in Dc it occurred between the 4th and 8th instars (Fig. 3). Although some Dh individuals reached maturity earlier in the fish medium than in the control (4th instar; Fig. 3A and 3B), the variance of AFR was greater in the fish treatment, but only at the low algal food concentration (Fisher’s F Distribution, Table 4). Both Daphnia species had a greater clutch size vs. body length in fish medium compared with the control (Table 3, ANCOVA). In Dc, fish medium (1) increased the integrated egg and body volume of lipid reserves in the first-clutch females (Table 2), (2) increased the level of lipid reserves (comparison of elevations) and (3) enhanced the rate of increase in lipid reserves from the instar with the minimum volume of such reserves until first reproduction (comparison of slopes, Table 3). In Dh, it lowered the level of lipid reserves, but only at high bacterial and low algal food concentrations (ElowB, Table 3, comparison of elevations).

The effect of bacterial food was generally more apparent at low than at high algal food concentration (Table 2, B × Sc interaction), and seemingly more evident in Dc (Figs. 2, 3 and 4). When the effect of kairomones prevailed over the effect of fish-related bacterial food, the cumulative effect of fish medium was more apparent at high than at low algal concentration (e.g., Fig. 1).
The effect of fish-related bacterial food \((F_B \times B)\) interaction was generally greater under low algal food conditions and more apparent in \(Dc\). Such an effect may consist of either an increase or a decrease in the difference between fish medium and the control at the different bacterial concentrations. In both \(Daphnia\) species, fish-related bacterial food influenced GR (Table 2), the clutch size versus body length at first reproduction (Table 3), and the accumulation of lipid reserves from the minimum level until first reproduction (Table 3). The difference in GR between control and fish medium was negligible in \(Dh\) and negative in \(Dc\) at high bacterial abundance \((E_{\text{high}})\), but this changed to positive values under intermediate \((E_{\text{m}})\) and low \((E_{\text{low}})\) bacterial food conditions (Fig. 1). In both species, the difference in clutch size on body length was greatest at high \((E_{\text{high}})\), lower at intermediate \((E_{\text{m}})\) and lowest at the low \((E_{\text{low}})\) bacterial food level, but only at the low algal food concentration (Table 3).

In \(Dh\), the difference in the accumulation of lipids from the minimum level until first reproduction was highest at the low bacterial food level (Table 3, comparison of elevations in \(E_{\text{low}}\) at low algal food concentration). The direction of this difference was reversed in \(Dc\) (Table 3, Fig. 4). Moreover, only in \(Dc\) did the effect of fish-related bacterial food influence (1) SFR, (2) AFR, (3) variance in AFR, (4) the number of pre-adult instars, (5) egg volume in the first-clutch females, and (6) the integrated egg-body volume of lipids. In SFR, with reducing bacterial food concentration (from \(E_{\text{high}}\) to \(E_{\text{low}}\)), the difference increased at high (diff. \(-0.01, 0.02, 0.04\) mm, Fig. 2A) and decreased at low (diff. \(-0.03, 0.02\) and \(0.01\) mm, Fig. 2B) algal food concentration (significant \(F_m \times B \times S_c\) interaction). The difference in AFR (Fig. 2), the number of pre-adult instars (Figs. 2 and 3), and the integrated egg-body volume of lipids (Fig. 4) was large at high \((E_{\text{high}})\) and decreased at the intermediate \((E_{\text{m}})\) to low \((E_{\text{low}})\) bacterial food level. The difference in egg volume was relatively low at high \((E_{\text{high}})\), slightly higher at intermediate \((E_{\text{m}})\), and much higher at the low \((E_{\text{low}})\) bacterial food level. Fish-related bacteria lowered the variance in AFR (Fig. 3) and that of the second reproduc-
Fig. 2. Instar-to-instar mean (±1 SD) body length increase in *D. hyalina* (*Dh*, upper panel) and *D. cucullata* (*Dc*, lower panel), from a neonate (0.00–0.25 d) until release of the first neonates (females with the second clutch of eggs), in fish medium (red line) and the control (black line), at high (1.0 mg C/L, panel A) and low (0.1 mg C/L, panel B) algal food concentrations and at different levels of bacteria as an additional food: high (*E_{high})*, medium (*E_{mid}*) and low (*E_{low})*. It was assumed that the body length of a freshly molted *Daphnia* would not change until the next molt (body length of each animal was checked again at that time).
tion, thus increasing the difference in variance between fish medium and the control (Fisher’s F Distribution, Table 4, E<sub>low</sub>).

**Experiment with kairomones alone**

(***negligible effect of bacteria, E<sub>low</sub>*)

Analysis of the data from E<sub>low</sub> alone showed that both GR and SFR (Figs. 1 and 2) were (1) lower in the presence of kairomones (F<sub>k</sub> factor) than the control, (2) higher at high, than at low algal food concentration, and (3) higher in Dh than Dc (Table 5). Although the stronger effect of kairomones at the high algal food concentration (F<sub>k</sub> × Sc interaction) was not related to the *Daphnia* species (non-significant interaction F<sub>k</sub> × Sp), the effect of algae was stronger in Dh than in Dc (Table 5, Sc × Sp). The AFR and the number of pre-adult instars were affected by each of the three factors, but neither the F<sub>k</sub> × Sc nor the F<sub>k</sub> × Sp interactions (Fig. 3, Table 5). The effect of algal food concentration on AFR was higher in Dh than in Dc (Table 5, Sc × Sp).
Table 4. Fisher’s F distribution (F, p, df) for differences in the variance of age at first reproduction (Fig. 2) for D. hyalina (Dh) and D. cucullata (Dc) in fish medium and the control, at high (1.0 mg C/L) and low (0.1 mg C/L) algal food concentrations (indicated in the top row of column headings) and at different levels of bacteria as an additional food (indicated in the second row of column headings): high (Ehigh 0.07 mg C/L in control and 0.33 mg C/L in fish medium), medium (Emed 0.01 mg C/L in control and 0.10 mg C/L in fish medium), and low (Emed 0.01 mg C/L in control and 0.01 mg C/L in fish medium). Statistical significance is accepted after Bonferroni’s adjustment for 12 comparisons at *p < 0.00427, **p < 0.00043 or ***p < 0.00004. NS indicates no significance. In each comparison, df is shown for the control (C) and fish (Fm) treatment.

<table>
<thead>
<tr>
<th>Species</th>
<th>High</th>
<th>Medium</th>
<th>Low</th>
<th>High</th>
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<tr>
<td>Dc</td>
<td>5.54</td>
<td>***</td>
<td>31:31</td>
<td>2.22</td>
<td>NS</td>
<td>25:26</td>
<td>0.99</td>
<td>NS</td>
<td>21:22</td>
</tr>
</tbody>
</table>

The results of this study indicate that compounds released by planktivorous fish have a dual effect on Daphnia. First, through chemical information on predation risk they trigger a phenotypic response to that risk. Second, through the provision of additional high-quality food they stimulate growth and fecundity, and assure higher survival when food is limiting. Our experiments also show that the smaller Daphnia cucullata (Dc) has a greater ability to retain fish-related bacterial food than D. hyalina (Dh), due to the smaller filtering mesh size of its juvenile stages. At first glance, such an ability could easily be regarded as a consequence of being smaller. However, it is equally possible that it stems from the need to avoid resource competition with larger-bodied Daphnia by broadening their feeding niche through the inclusion of small particles of high-quality food into their diet, i.e., particles that are not retained by coarser filters. Our results also suggest that the real effect of fish kairomones can only be determined when the effects of fish-related bacteria (degradation of kairomones and obscuring their effects) have been excluded.

The abundance of bacteria in both the fish medium and the control was assumed to be dependent on the mesh size of filters used in preparation of these media which altered the amount of this additional food and its impact on Daphnia growth rate and life history parameters. Our findings demonstrate that fish-related bacterial food enhance Daphnia growth and reproduction. This was more apparent in the smaller-than the larger-bodied Daphnia species especially under low algal food conditions. There are two likely reasons why smaller Dc may use a ‘bacterial treat related to fish’ more efficiently. First, this may result from greater efficiency in retaining bacterial food by the finer mesh of its filters, especially in juveniles (see Geller and Müller 1981 and The filtering apparatus in the Results). This explanation is supported by the higher biomass of bacterial food found in the fish medium than the control. Second, this may be the result of the greater phosphorus demand observed in smaller zooplankton species (Elser et al. 2000, DeMott and Pape 2005), that should fare better when resources are P-enriched (Sterner...
This explanation is supported by the results of earlier studies (Hessen and Andersen 1990, Sterner and Elser 2002) and by this study that mass- or carbon-specific P content (C:P ratio) was 10-fold lower in bacteria than in algae. The greater ability of Dc to benefit from fish-related bacterial food can be regarded as a result of its temporal and spatial adaptation to coexist with planktivorous fish. A possible temporal

Fig. 4. The evolution of lipid reserves from the initial use of natal reserves, through the accumulation of stored lipids before first reproduction (when the first eggs appear in the brood chamber), in D. hyalina (Dh, upper panel) and D. cucullata (Dc, lower panel) in the fish medium (red line) and the control (black line), at high (1.0 mg C/L, panel A) and low (0.1 mg C/L, panel B) algal food concentrations and at different levels of bacteria as an additional food: high (E<sub>high</sub>), medium (E<sub>m</sub>) and low (E<sub>low</sub>). Individual lipid reserves are shown as the ratio (mean ± 1 SD) of the integrated volume of lipid droplets (mm<sup>3</sup>) to body mass (mg body carbon). Estimates were made every 8 hours for each freshly molted individual.
adaptation is increased efficiency in utilizing nanoplanktonic food when fish are abundant. Such an adaptation could give smaller Daphnia a competitive advantage, and (together with their lower vulnerability to visually hunting predators, Gliwicz 1969) can lead to their dominance over large-bodied species. This is consistent with the results of Iwabuchi and Urabe (2010), who demonstrated that small cladocerans such as Ceriodaphnia quadrangula can outcompete larger Daphnia pulex even in the absence of size-selective predation due to their greater ability to benefit from P-rich bacterial food. This is also consistent with the more recent finding by Iwabuchi and Urabe (2012) that the quality of food influences the competitive superiority of a herbivore zooplankton species in terms of threshold food levels. A possible spatial adaptation of Dc is its increased efficiency in retaining the smallest food particles on fine mesh size filters in warm, and hence less viscous (Abrusan 2004) subsurface water layers during the summer stratification.

The negligible effect of a ‘bacterial treat’ when it co-occurs with a strong response to the fish medium in Dh, confirms the validity of our assumption that the effect of the fish threat was equally strong in each experiment despite the different biomass of bacteria present. Therefore, it may be speculated that the effect of fish-related bacteria on Dc (increased or decreased difference between the fish medium and control) was due to obfuscating the effect of kairomones rather than their degradation.

When the two effects, i.e., that of fish kairomones and that of fish-related bacteria, work in the same direction to cause a change in life history parameters, even a slightly higher density of additional bacterial food related to fish should increase the significance of the difference between the fish and control media. In our study, this was the case when each of the two effects (1) accelerated maturity, (2) increased variability in the age at first reproduction, (3) caused a decline in the number of pre-adult instars, and (4) resulted in increased clutch size vs. body length regressions. This suggests that fish-related bacterial food may have caused an overestimation of the real effect of kairomones on these parameters in some previous studies (e.g., Weider and Pijanowska 1993, Reede and Ringelberg 1998).

Conversely, if the two effects work in the opposite direction, the outcome produced by a higher density of fish-related bacterial food seems to be more complex as it may lead to either a decrease or an inversion of the differences between the fish medium and the control. When the kairomone effect prevailed over that of the bacterial food in each of the three bacterial food levels employed, the inter-treatment difference (between the fish medium and the control) in life history parameters was reduced. This was the case when kairomones caused a decline and

Table 5. Three-way ANOVA (F, p and df) to test the significance of the effect of fish kairomones (Fk), algal food (Sc), Daphnia species (Sp, D. hyalina and D. cucullata), and interactions between these factors in experiment at low bacterial levels (Eb), on individual growth rate from newborn neonates to the ovigerous adults (GR), size at first reproduction (SFR), age at first reproduction (AFR), the number of pre-adult instars (Instars), minimum volume of body lipid reserves (Juvenile), integrated egg and body volume of lipid reserves in the first-clutch females (Adult), and egg volume. Statistical significance is accepted at *p < 0.05, **p < 0.005 or ***p < 0.0005. NS indicates no significance.

<table>
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<tr>
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<th>GR</th>
<th>SFR</th>
<th>AFR</th>
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<th>Juvenile</th>
<th>Adult</th>
<th>Egg volume</th>
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<td></td>
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<td>5</td>
</tr>
<tr>
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</tr>
<tr>
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<td>4</td>
<td>NS</td>
<td>1</td>
<td>NS</td>
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</table>

Notes: Error degrees of freedom: GR, 44; SFR, AFR, instars, 182; juvenile lipid volume, 220; adult lipid volume and egg volume, 192.
bacterial food caused an increase in (1) growth rate in Dh, (2) egg volume in Dc, and (3) rate of lipid accumulation in the course of the ontogenetic change from the instar at a minimum lipid volume to the instar at 1st reproduction at the highest bacterial level, the higher density of fish-related bacterial food caused an increase in growth rate and size at first reproduction in Dc. The decrease or inversion of the differences demonstrate that additional bacterial food related to fish could have caused under- rather than over-estimation of the real effect of kairomones on these parameters in some previous studies.

We suggest that the mis- (either over- or under-) estimation of the real effect of kairomones might have been an issue in some earlier kairomone studies performed with fish media and media containing chemical cues from invertebrate predators (e.g., Chaoborus, Notonecta and Triops). In particular, this may be the case in the studies where (1) media were prepared with lake water that had been filtered through filters with mesh sizes coarser than 0.2 μm, (2) bacterial abundance was not rigorously controlled with antibiotics, (3) treatments were made with algal concentrations well below the incipient limiting level, and (4) cladoceran species such as D. cucullata and D. magna, which are able to benefit from bacterial food, were used (Geller and Müller 1981, and Gophen and Geller 1984). A misestimation of the real effect of kairomones might also have occurred in studies comparing the response of Daphnia to the fish medium from lakes with and without planktivorous fish, which showed a smaller effect in species from the former (Machácek 1993, Boersma et al. 1998, Gliwicz and Maszczyk 2007). Our findings suggest that the observed differences may not only be due to a weaker response to predation, but could also result from a greater ability to retain the smallest food particles (Koza and Kořínek 1985, Lampert 1994, Lampert and Brendelberger 1996). Furthermore, a similar misestimation could possibly have occurred in studies comparing the reaction to fish medium of clones from different habitats, like that of Boersma et al. (1998), who showed that clones of D. magna differ widely in their response to fish medium. Our results show that such variation could stem not only from differences in their sensitivity to kairomones, but also from their different abilities to benefit from fish-related bacterial food. Finally, such misestimation could also have occurred when response of Daphnia to negative size-selective predation (e.g., invertebrate predation) was tested (Spitze 1991, Jeyasingh and Weider 2005). In those cases selective pressure was found to favor more rapid attainment of a safe body size, thus promoting efficient exploitation of P-rich food (e.g., predator-related bacteria).

The need to separate the effect of chemical information from the effect of food level may be crucial not only in the case of predator-released compounds. It is also important in the case of substances related to the prey. They not only carry information on prey location (foraging kairomones), but may also be used as a source of food. Recently, Weinhold and Baldwin (2011) revealed that a ground-hunting predator, the omnivorous ant (Pogonomyrex rugosus), uses volatile branched-chain aliphatic acids to locate their prey (caterpillars), whereas an obligatory predator of the same prey, the big-eyed bug (Geocoris spp.), does not. In this case, infochemicals could themselves be a source of important nutrition or they may be associated with exudates and feces that improve nutritional conditions for the omnivorous predator.

In our study, the treatment where bacterial abundance in the media was reduced by the utilization of the finest (0.2 μm) filter (E100) revealed the effect of kairomones alone. Such an approach allowed us to attain a similar (difference < 4%) and reasonably low (<10% of total POC bound up in algae and bacteria) biomass of bacteria in both the fish medium and the control (Table 1). The effect of kairomones was similar to that recorded in the majority of previous studies examining the influence of fish medium on the growth rate and life history of Daphnia (Riessen 1999, Lass and Spaak 2003). The presence of kairomones resulted in reductions in growth rate, size at first and second reproduction, egg volume (Machácek 1991, Stibor 1992, Weider and Pijanowska 1993), and caused a decline in the egg and body integrated lipid volumes in first-clutch females. Furthermore, kairomones produced (1)
accelerated maturation, (2) an increased number of eggs per clutch vs. body length (e.g., Machacek 1991, Stibor 1992), and (3) a reduced number of pre-adult instars (Sakwinska 2002). Although the results we have obtained are in accordance with those of the previous studies indicated above, the strength of the significance between different algal food conditions, species, and within parameters is more realistic because of the semi-controlled abundance of fish-related bacterial food.

The separation of the effect of kairomones from the effect of fish-related bacterial food could also provide an explanation for some of the inconsistencies found in reports on the effects of predator cues at different concentrations of algal food. While the majority of investigations on the morphological (Wiackowski and Szkarlat 1996), life history (Walls et al. 1991), growth rate (Weetman and Atkinson 2002) and behavioral adjustments (Hays et al. 2001) of Daphnia show weaker reactions to kairomones at low food levels, some have not confirmed a synergistic effect (Doksaeter and Vijverberg 2001), and a few observed enhanced anti-predator defenses (Reede and Ringelberg 1995, Weber 2001, Pauwels et al. 2010). In our study, the effect of kairomones alone appeared to be weaker at lower than at high algal food concentration. This indicates that the effect of fish-related bacterial food may lead to more significant misestimation of the exclusive effect of kairomones when other food is limited.

When the effect of additional bacterial food was excluded, the effect of kairomones appeared to be equally strong for both Daphnia species. Thus, our results do not support two important claims made previously in literature. First, the effect of fish kairomones was not stronger in larger zooplankton species known for their greater susceptibility to size-selective visual predation (Boersma et al. 1996, Spaak et al. 2000). Second, it was not weaker due to the insufficient reduction of the risk of predation by life history adjustments (Sakwinska and Dawidowicz 2005). Removing the effect of fish-related bacteria allowed us to detect the costs of inducible defenses represented by the lower rate of lipid accumulation until the time of first reproduction. Such a reduction was apparent only in Dh at the low algal concentration (Fig. 4B) when gains from the exploitation of fish-related food appear to be lower than the costs of the induced defenses. In some previous kairomone studies, predator-related food may have hidden the real costs of antipredation defenses such as those arising from diel vertical migrations of Daphnia exposed to fish (Dawidowicz and Loose 1992), neck teeth formation in Daphnia exposed to Chaoborus (Spitze 1992, Tollrian 1995), or posterolateral spine formation in Brachionus calyciflorus in response to Asplanchia (Gilbert 1980).

Although we could exclude the effects of fish-related bacterial food to successfully investigate the effect of kairomones alone on Daphnia growth rate and life history, we were unable to examine the effect of a fish-related bacterial ‘treat’ while excluding the effect of kairomones. This might be more easily achieved when the true nature of kairomones has been determined. Once their chemical identity is known, the quantitative (and qualitative) effects of kairomones may be more readily accounted for. Therefore, elucidation of the true nature of kairomones through detailed and sophisticated studies employing advanced analytical techniques remains an important goal for ecological studies (Pohnert et al. 2007, Akkas et al. 2010).

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LITERATURE CITED


