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Does ultraviolet radiation alter kairomones? An experimental test with *Chaoborus obscuripes* and *Daphnia pulex*

BARBARA STERR AND RUBEN SOMMARUGA*

LABORATORY OF AQUATIC PHOTOBIOLOGY AND PLANKTON ECOLOGY, INSTITUTE OF ECOLOGY, UNIVERSITY OF INNSBRUCK, TECHNIKERSTR. 25, 6020 INNSBRUCK, AUSTRIA

*CORRESPONDING AUTHOR: ruben.sommaruga@uibk.ac.at

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The kairomone released by Chaoborus (phantom-midge) larvae induces neck protuberances (neckkeel with neckteeth) in the dorsal anterior margin of the head of Daphnia that are part of the defense response of this cladoceran against predation pressure. In aquatic ecosystems, kairomones are exposed to different environmental factors that may affect their fate. Among them, solar ultraviolet radiation (UVR) is known to alter chemical moieties and degrade organic substances. In this study, we tested the hypothesis that UVR alters kairomones reducing their efficiency as infochemicals. A kairomone extract from Chaoborus obscuripes pre-exposed to UVR for 5 or 10 h was assessed for the induction of neck protuberances in Daphnia pulex and compared with a control (i.e. with the kairomone unexposed to UVR). The results indicated that after 5 h exposure to UVR, the kairomone was photoaltered and the formation of neck protuberances strongly reduced (number of neckteeth by 31% and height and with of neckkeel by 55 and 43%, respectively). Exposure of the kairomone for additional 5 h did not result in further reduction of neck protuberances. This is the first report indicating that UVR has the potential to alter infochemicals in the aquatic realm and thus, to indirectly affect predator–prey interactions.

INTRODUCTION

In aquatic ecosystems, defenses induced by chemical cues are common in vertebrates, invertebrates and plants influencing their morphology, behavior and life history (Harvell and Tollrian, 1999). Such inducible defenses are triggered by infochemicals called kairomones released by predators and competitors (Harvell and Tollrian, 1999; Tollrian and Dodson, 1999). Kairomones are allelochemicals interacting between individuals of different species and benefiting the receiver (i.e. prey). The effects of kairomones have been well studied in the predator–prey system of the phantom-midge larvae *Chaoborus* and the water flea *Daphnia* (Tollrian and Dodson, 1999). In the presence of the kairomone from *Chaoborus*, juveniles of *Daphnia* primarily in the second instar develop a neckkeel with so-called "neckteeth" on the dorsal anterior margin of the head (Krueger and Dodson, 1981; Tollrian, 1993).

The production of neckteeth is concentration-dependent and follows a saturation curve (Tollrian, 1993), but its expression varies among *Daphnia* clones (Havel, 1985; Parejko and Dodson, 1991; Spitze, 1992). Stabell *et al.* (Stabell *et al.*, 2003) suggested that the induction of defenses in *Daphnia* is a reaction to dormant conspecific alarm signals triggered by enzymes present in predators or in the water. Laforsch and Tollrian (Laforsch and Tollrian, 2004) argued that significant changes in the neck region induced by predator-released kairomones can only be observed in the last embryonic stage of *Daphnia* and proposed that kairomones are detected by chemosensillae located at the tip of the first antennae. Despite many efforts to chemically characterize kairomones, their exact

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chemical structure remains unknown, but the kairomone from *Chaoborus* is a low-molecular-weight (<500 Dalton) water soluble non-protein substance with hydroxyl and carboxyl groups (Hebert and Grewe, 1985; Tollrian and von Elert, 1994).

Infochemicals released into aquatic ecosystems are exposed to several processes that may affect their fate such as bacterial degradation, turbulence and physicochemical alteration (Tollrian and Dodson, 1999). However, Tollrian and von Elert (Tollrian and von Elert, 1994) showed that the kairomone from *C. flavicans* is stable under high temperature and pH conditions.

The effect of ultraviolet radiation (UVR, 280–400 nm) on kairomones has not been assessed so far; however, it is well established that UVR causes not only negative effects on aquatic organisms, but it also plays an important role in altering and degrading dissolved organic substances (Wetzel, 2003). Solar UVR can also induce the production of reactive oxygen species (ROS) that alter the original function of chemical substances (Kieber *et al.*, 2003 and references therein). Therefore, establishing whether UVR affects the function of kairomones will add to our understanding of the fate of these infochemicals in aquatic ecosystems.

In this study, we tested the hypothesis that UVR alters kairomones and thus, reduces the inducible defense of the prey. This test was done by exposing the kairomone from *C. obscuripes* (van der Wulp 1858) to artificial UVR and assessing the induction of the neck region in *D. pulex* (Leydig 1860).

METHOD

Cultivation conditions of *D. pulex*

The cladoceran *D. pulex* is widespread in the Holarctic and Neotropic temperate regions and is a common inhabitant in small eutrophic ponds. For the experiments, *D. pulex* was collected from a small artificial eutrophic pond (depth: ~2 m) located near the University of Innsbruck (47°15′N, 11°20′E) by vertical tows with a plankton net (55 μm mesh size). This fishless pond contains different *Chaoborus* species present in high densities. Thus, the original population of *D. pulex* has pronounced neck protuberances in the dorsal region.

To reduce the number of neckteeth in *D. pulex*, cladocerans were cultivated in filtered (50 μm) aged rainwater at densities of 10 individuals L^{-1} in 1 L glass jars for several generations. The water was exchanged at least once a week and the organisms were fed with *Chlorella* cf. *minutissima* (every 2–3 days) at concentrations of ca. 1 mg C L^{-1}. This food level was above the limiting POC level known for *Daphnia* (Carotenuto and Lampert, 2004). Cultivation and also experiments were conducted in a climate chamber at ~22°C with a light:dark cycle of 14.5:9.5 h. At the beginning of the kairomone bioassay, the culture was 4 months old.

Cultivation conditions of *Chlorella cf. minutissima*

The green alga *Chlorella cf. minutissima* was cultured in Woods-Hole MBL-medium (Guillard and Lorenzen, 1972). Every 2 weeks, 10 mL of the culture was inoculated into 90 mL autoclaved fresh medium and cultured in a climate chamber as described earlier for *D. pulex*. To follow changes in the POC content of the algal culture, samples of 10 mL were collected every second day and filtered through a muffled (450°C, 4 h) glass fiber filter (Whatman GF/F 25 mm). Then, the filter was dried at 50°C for at least 24 h and the carbon content was measured with a CHN-Analyzer (Thermo Flash EA 1112, Thermo Finnigan).

Kairomone extraction and enrichment

To obtain the kairomone from *C. obscuripes*, large numbers of larvae mainly from the fourth-instar were collected on two consecutive days in summer from the same artificial pond as *D. pulex* by horizontal tows as described earlier. This was done to eliminate the potential changes in the amount of kairomone present. Then, the larvae of *C. obscuripes* were sorted out manually under a stereomicroscope and placed in the synthetic RT-medium described by Tollrian (Tollrian, 1993) for 24 h at a density of 500 individuals L^{-1}. This medium mainly consists of inorganic chemicals and it has a pH of 7.9 and a conductivity of 220 μ S cm^{-1} at 25°C. We used the RT-medium for the kairomone enrichment, because it does not favor bacterial growth (Tollrian and von Elert, 1994). During the first incubation in this medium, the larvae were fed *ad libitum* with *D. pulex* from the stock culture (Tollrian and von Elert, 1994). Then, the larvae were transferred into filtered [0.2 μm surfactant free cellulose acetate (SFCA)-filter, 25 mm, Nalgene] RT-medium (not older than 3 days) and incubated again for 24 h without feeding (Tollrian, 1993; Tollrian and von Elert, 1994).

After the second incubation, the larvae were removed with a 50 μm mesh plankton net and the kairomone-conditioned medium was stored at −80°C. After 18 days, the medium was thawed and the kairomone was concentrated using cartridges filled with C_{18}-(Octadecyl)-silica-bonded sorbents (Whatman) that

were prepared with five bed volumes of methanol (99.9% HPLC gradient) and of Milli-Q water (Tollrian and von Elert, 1994). Before starting the extraction, methanol was added to the medium at a final concentration of 1% (v/v) and then filtered through a 0.2 μm pore size SFCA-filter. After filtration, the cartridges were washed with 5 mL of Milli-Q water and then desorbed by 10 mL methanol (99.9% HPLC gradient). The resulting extract was dried in a SpeedVac at room temperature, and then resuspended with 10 mL Milli-Q water. Finally, the kairomone extract was distributed into sterile vials under a laminar flow chamber and then stored at −80°C.

UVR exposure of the kairomone-extract

To test the effect of UVR on the kairomone, part of the extract (i.e. from the same extraction time as above) was thawed at room temperature and transferred into sterile 80 mL quartz tubes (diameter: 25 mm; length: 137 mm). Then, the extract was exposed to artificial UVR and PAR for 5 or 10 h in a walk-in chamber at 17 ± 1°C. After exposure, the extract was distributed into 2 mL sterile Eppendorf vials under a laminar flow chamber and stored frozen at −80°C for 1 month. The UVR source consisted of four UV-A-340 (Q-Panel Co., Cleveland, OH, USA) lamps with a maximum emission at 340 nm and producing no radiation below 280 nm. The integrated irradiance between 280 and 320 nm was 1.4 W m^{-2} corresponding to a final dose of 25.2 kJ m^{-2} for 5 h exposure, which is equivalent to a typical daily integrated value for summer at mid latitudes. PAR was provided by two white fluorescent tubes (cool white L36/ W20, Osram) emitting 80 μmol quanta m^{-2} s^{-1}. During a preliminary trial, this PAR irradiance did not have a negative effect on the kairomone (i.e. no reduction in neck protuberances was observed, data not shown). The spectrum of the lamps was measured with a double monochromator spectroradiometer (Sommaruga *et al.*, 1996). During exposure, all quartz-tubes were kept in a water bath to keep temperatures within ± 1°C at a distance of 25 cm from the lamps.

Experimental design

To ensure the complete development of the *Daphnia* embryos in the presence of the kairomone and also to reduce the mortality rate during the experiments, mature *D. pulex* with a body length between 1250 and 1500 μm were haphazardly selected from the stock culture. We first tested whether the extracted kairomone was able to induce neck protuberances in *Daphnia* by comparing the response to a control without kairomone. Then, to test for the effect of UVR on the kairomone, the experimental design included the following treatments with five replicates each: (i) unexposed kairomone (Control), (ii) kairomone exposed to UVR+PAR for 5 h (+ Kairo: 5 h), and (iii) kairomone exposed to UV+PAR for 10 h (+ Kairo: 10 h). Each replicate of the treatments consisted of one *Daphnia* mother individual placed in a well with 1.5 mL double-filtered (0.2 μm SFCA-filter) rain water together with 0.5 mL of *Chlorella* culture corresponding to ∼2 mg C L^{-1}, plus 0.5 mL of the kairomone extract. In the control, 2.0 mL of double-filtered rain water was added to reach the same final volume as in the treatments. The volume of kairomone extract was selected based on a previous trial using two different volumes (i.e. 0.5 and 1.0 mL) to determine the maximum induction of neckteeth. The *Daphnia* mothers were transferred daily into fresh medium and the kairomone extract was added or not, until they released their offspring. Afterwards, the neonates were measured by optical microscopy without using fixatives.

The following parameters were assessed on the same day in neonates of the first clutch: the number of neckteeth (*n*-NT), the height (h-Nk) and length (l-Nk) of neckkeel, and the height (h-NT) and width (w-NT) of neckteeth. Additionally, the body length was also measured to control for the size of the first clutch. The body length was measured from top of the head to the base of the tail at 100x and the neckteeth and neckkeel at 400x. The average body length of the juvenile daphnids was 666.76 μm (± 27.05 μm). The number of *Daphnia* neonates measured in a single clutch was variable but ranged from 4 to 7, thus data for the different parameters were combined.

Statistical analyses

The statistical tests were conducted with Sigma-Stat (Systat, Software Inc., San Jose, CA). Results from the first experiment were tested with a Student's *t*-test, whereas in the second experiment addressed to assess the effect of UVR on the kairomone, significant differences among treatments were tested by one-way-ANOVA. The *post hoc* comparisons were tested by the Holm-Sidak method with an overall significance level of 0.05.

RESULTS

The addition of unexposed kairomone caused a significant increase in the number of neckteeth, height and length of the neckkeel, and in the height and width of the first two neckteeth, indicating that the extraction was successful (Table I and Fig. 1). The first clutch of *D. pulex*

in the control without kairomone showed a maximum induction of two neckteeth, whereas in the presence of unexposed kairomone, up to four neckteeth were observed. Moreover, the first and the second neckteeth showed a significant increase in height and width (*t*-test, Table I) under the presence of unexposed kairomone.

The exposure of the kairomone to UVR caused a significant decrease in the height and width of the neckkeel (ANOVA, $P < 0.05$, Table II and Fig. 1) and also reduced the average number of neckteeth, though this effect was marginally not significant ($P < 0.06$, Table II). There was not only a reduction in the formation of the neckkeel, but also in the height and particularly in the width of the neckteeth (Fig. 2). Although the height of the first neckteeth decreased, this was not significant in all treatments, but the width declined significantly in the treatment where the kairomone was exposed for 5 h to UVR ($P = 0.006$; Fig. 2). In contrast to the effect of irradiated kairomones on the first neckteeth, the height of the second neckteeth (Fig. 2) decreased significantly already in the treatment where the kairomone was exposed for 5 h to UVR ($P = 0.031$). This was also true for the width of the second neckteeth, but this change was again not significant, as well as for the height and the width of the third and forth neckteeth (data not shown). Exposure of the kairomone extract for 5 or 10 h did not cause significantly different effects (Table II and Fig. 2).

DISCUSSION

Effect of UVR on kairomones

In aquatic ecosystems, infochemicals such as kairomones are subjected to the action of several environmental factors that may alter their chemical integrity and therefore their function. Among these factors, changes in pH and temperature, but also in turbulence, water chemistry and light have been proposed (Tollrian and Dodson, 1999). In fact, very little is known about how stable kairomones are and what their half-lives under natural conditions are. One of the few studies available on this topic is that of Tollrian and von Elert (Tollrian and von Elert, 1994). These authors assessed the effects of pH and temperature on the kairomone activity of *C.*

Table I: Mean ± 1 SD values for the neck protuberances induced in Daphnia pulex by the unexposed kairomone (+ Kairomone) in comparison to the control without kairomone (− Kairomone)

	Treatment		
Parameter	−Kairomone	+Kairomone	*P*-value
Number of neckteeth (NT)	1.33 ± 0.52^a	2.75 ± 0.96^b	0.015
Height of neckkeel (μm)	2.50 ± 0.00^a	9.06 ± 2.77^b	<0.001
Length of neckkeel (μm)	28.54 ± 3.74^a	54.38 ± 12.97^b	0.001
Height of first NT	3.54 ± 0.94^a	8.44 ± 1.20^b	<0.001
Width of first NT	7.71 ± 1.46^a	10.63 ± 1.25^b	0.012
Height of second NT	0.63 ± 1.05^a	6.25 ± 1.02^b	<0.001
Width of second NT	0.63 ± 1.05^a	6.25 ± 1.44^b	<0.001

Different letters for a parameter indicate significant differences between treatments.

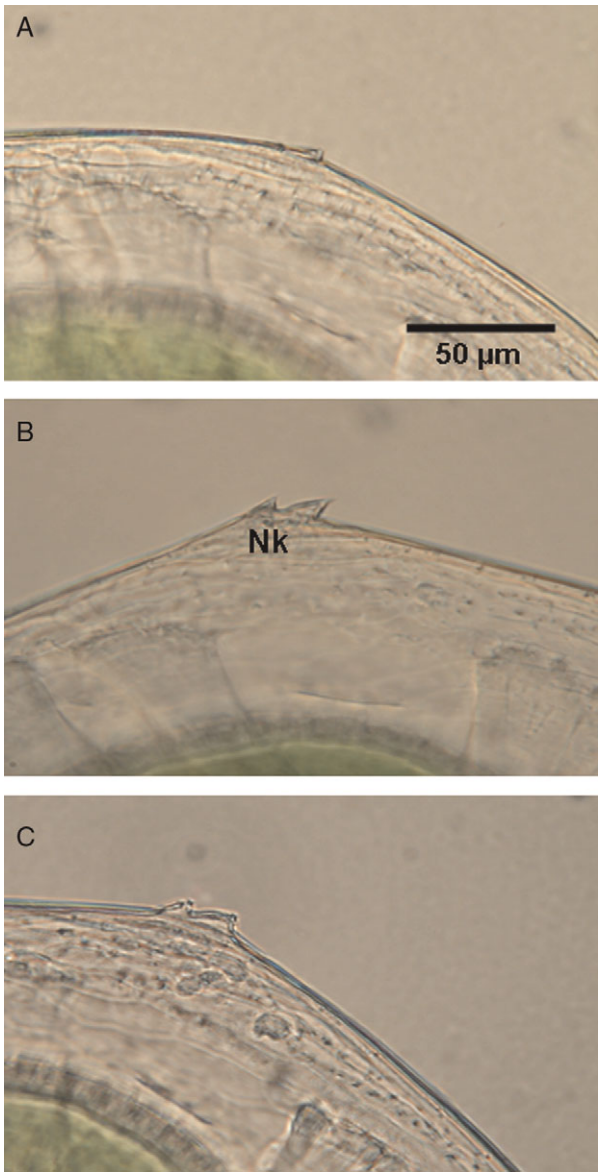

Fig. 1. Micrographs of the neck region of *Daphnia pulex* showing the protuberances of individuals cultured in the absence of kairomone (**A**), in the presence of unexposed kairomone (**B**), or kairomone exposed to artificial UVR (**C**). Note the different height and width of the neckkeel (Nk) in the three treatments. Scale bar is the same for all micrographs.

flavicans and found that it is very resistant even when the extract is boiled and autoclaved for 20 min or exposed to low (2.0) and high pH (10.0) for 12 h.

Table II: Mean ± 1 SD values for the number of neckteeth and height and length of neckkeel induced in Daphnia pulex by unexposed (Control) and exposed kairomone for 5 h (+Kairo: 5 h) and 10 h (+Kairo: 10 h) to artificial UV radiation

	Treatment			
Parameter	Control	+Kairo: 5 h	+Kairo: 10 h	*P*-value
Number of neckteeth	2.75 ± 0.96^{a}	1.90 ± 0.99^{a}	1.93 ± 0.92^{a}	0.06
Height of neckkeel (μm)	9.06 ± 2.77^{a}	4.08 ± 1.92^{b}	5.00 ± 1.70^{b}	<0.001
Length of neckkeel (μm)	54.38 ± 12.97^{a}	31.00 ± 9.80^{b}	36.04 ± 12.81^{b}	0.011

Different letters for a measured parameter indicate significant differences between treatments.

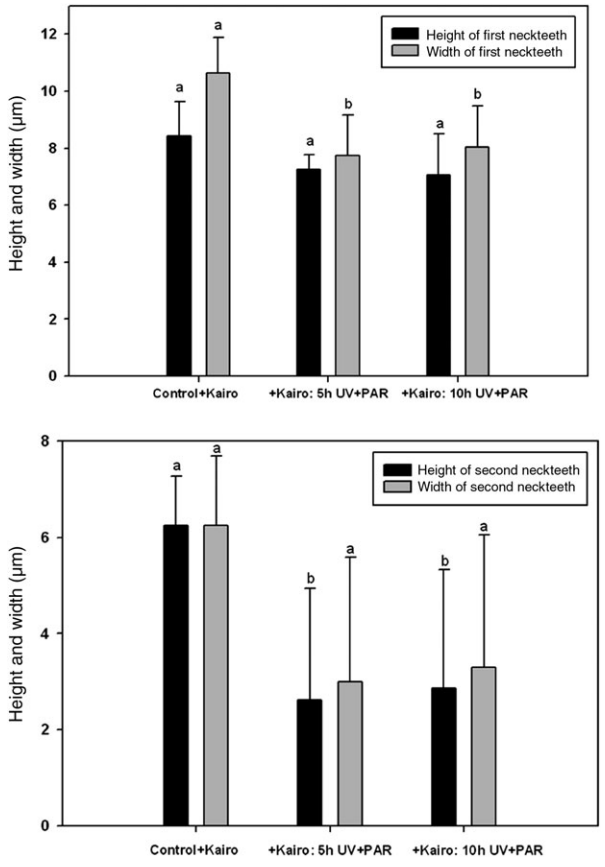

Fig. 2. Effect of the unexposed kairomone (Control) and kairomone exposed for 5 and 10 h to artificial UV radiation (+Kairo: 5 h, +Kairo: 10 h) on the height and width of first (upper panel) and second neckteeth (lower panel). Different letters within a parameter are used to indicate significant differences between treatments.

Our rationale for testing the effects of UVR on kairomones was the well established ability this environmental factor has to alter dissolved organic molecules. Among the many chemical processes influenced by UVR, Wetzel (Wetzel, 2003) describes, for example, the partial and complete photolytic degradation of dissolved organic matter (DOM), such as of humic substances, to CO and CO_2 (i.e. photochemical mineralization). The photochemical degradation of DOM, especially the loss of UV absorbing moieties (chromophores) of DOM by UVR is called photobleaching (Hargreaves, 2003). The results of the present study indicate that the kairomone integrity was negatively affected after short exposure (5 h) to UVR equivalent to the accumulated irradiance found on a sunny day in summer. This effect was translated into a significant reduction of the neck protuberances of *D. pulex*.

Our experiments were carried out with artificial UVR and PAR that do not entirely simulate the natural solar spectrum because the irradiance emitted by the lamps at wavelengths >340 nm (long UV-A wavelengths) is significantly lower. At present, it is unknown what kind of chromophore is involved in the UV absorption by kairomones and its spectral absorption characteristics. However, photobleaching of chromophoric DOM (CDOM) by natural sunlight is mainly caused by UV-A (315–400 nm) and blue light radiation (Osburn *et al.*, 2001), which were underrepresented in our experiments. Thus, it is possible that the negative effect on kairomones is much stronger under natural sunlight exposure.

In aquatic systems, UVR and PAR penetrate to different depths depending on the abundance of phytoplankton and concentration of suspended particles and CDOM (Hargreaves, 2003). As a result of the differential attenuation caused by CDOM, UV-A penetrates deeper in the water column than UV-B (280–315 nm) radiation, but less than PAR. Considering that photolytic processes are restricted to the penetration depth of UVR (Wetzel, 2003), we can anticipate that photoalteration of kairomones will be stronger in clear lakes low in CDOM (e.g. humic substances). Nevertheless in lakes with high CDOM concentration, seasonal variations in UVR penetration occur (Morris and Hargreaves, 1997). For example, these authors observed in three lakes from the Pocono Plateau, Pennsylvania, USA, a decrease in UVR penetration during early spring and late autumn and an increase during mid summer, whereby this effect was stronger for UV-B than for UV-A. Based on additional experimental evidence, Morris and Hargreaves (Morris and Hargreaves, 1997) concluded that the higher UVR transparency in summer is related to significant DOC photobleaching and photodegradation. Thus, it is feasible that even in high CDOM lakes, the effectiveness of kairomones in the upper water layers is diminished in summer.

Under this scenario, the occupation by *Daphnia* of deep layers in the water column, where kairomones are active (i.e. they can elicit the prey response), will be beneficial to reduce their vulnerability to *Chaoborus* predation. In fact, it is well known that zooplankters such as *Chaoborus* and *Daphnia* show a diel vertical migration (DVM) in lakes (De Meester *et al.*, 1999) with maximal population densities found during daylight in deep layers. DVM is induced by changes in light intensity (Ringelberg, 1964), but food availability and antipredator avoidance are known factors explaining this pattern (De Meester *et al.*, 1999). More recently, UVR has been identified as a factor explaining the surface avoidance of zooplankton during daylight (Alonso *et al.*, 2004; Hansson *et al.*, 2007). For example, in a UV transparent lake, the cladoceran *D. catawba* displayed a distinct avoidance of the surface waters (Leech *et al.*, 2005). Rautio *et al.* (Rautio *et al.*, 2003) tested whether UVR or predation pressure (*C. obscuripes* and a "water boatman" from the family Corixidae) influence the distribution pattern of *D. longispina* in a shallow (maximum depth: 50 cm) fishless pond in subarctic Finnish Lapland. They found that on sunny days, *D. longispina* migrates downward to a depth of 25 cm (maximum penetration depth of UVR was 29 cm) to avoid UVR. In contrast, on cloudy days, this species is found at the water surface and therefore responds more to predator pressure. All these studies clearly indicate that UVR is crucial in determining the behavioral response of *Daphnia* and that in the presence of invertebrate predators, zooplankton are confronted by a dual conflicting selective pressure that may result in narrowing their depth range distribution in the water column during the DVM (Boeing *et al.*, 2004).

Yet, based on our results, we suggest that the ultimate response of *Daphnia* to UVR is 2-fold, i.e. not only avoiding the surface to diminish UV damage but also reducing the predation mortality of juvenile forms by being in contact with active kairomones. Thus, the penetration depth of UVR and the concentration of active kairomones in the water column could represent a gradient that is recognized by *Daphnia* and determines the migration to depths where they are double protected.

Response variability in effectiveness of the kairomone

Considering that the kairomone extract was a concentrated solution, a stronger expression of the number of neckteeth was expected. The *Daphnia* mothers used for the experiments stem from one single clone cultivated for >10 generations. After this period, we expected to obtain a homogeneous response of the clones to the kairomone extract. However, the variability observed was in some cases high. Spitze (Spitze, 1992) assessing the genetic variance among clones of *D. pulex* found an important genetic variance in the expression of neckteeth among clones. Thus, this variance among clones could explain the variability of our results. However, not only clonal variance probably contributed to the variability of our results, but also the method used to prepare the kairomone and the conditions during the experimental test could contribute to a reduced effectiveness of the kairomone. Many studies have used a "standard" procedure to extract the kairomones from *Chaoborus* larvae such as the one proposed by Hebert and Grewe (Hebert and Grewe, 1985). These authors extracted kairomones by boiling the *Chaoborus* larvae. However, Tollrian and von Elert (Tollrian and von Elert, 1994) argued that this method introduces some problems such as thermal stability of the kairomone extract and an enhanced bacterial contamination of the extract. Therefore, to minimize these effects, Tollrian and von Elert (Tollrian and von Elert, 1994) developed a new protocol that uses a solid-phase extraction device to enrich and purify the kairomone released by *C. flavicans*.

During the preparation of the kairomones, feeding during the first incubation is one possible critical factor influencing the effectiveness of kairomones. The induction of the neckteeth depends on the kairomone concentration (Tollrian, 1993) and also, the release of the kairomone from *Chaoborus* depends on the active ingestion of daphnids (Krueger and Dodson, 1981). Tollrian and von Elert (Tollrian and von Elert, 1994) did not mention the number of young daphnids used for feeding *Chaoborus* in their study, but a low abundance could be a reason for the apparent low kairomone effectiveness found in our experiments.

Tollrian and Dodson (Tollrian and Dodson, 1999) argued that bacterial degradation of kairomones is another possible factor influencing their effectiveness. In our study, all preparations and handling during the test were conducted under sterile conditions. However, during the first incubation, the kairomone was probably in contact with bacteria associated to *C. obscuripes* and also, during the experiment the presence of bacteria associated to *D. pulex* cannot be excluded.

For the experiments, we tried to obtain a clone of *D. pulex* without neckteeth. However, even if *D. pulex* was cultivated in rain water to eliminate the contact with kairomones, the clone had up to two neckteeth after the several generations. This means that the loss of neckteeth in *D. pulex* either takes longer or they retain a basal expression of this antipredator strategy.

Perspectives

Tollrian argued based on own unpublished data that a rapid degradation of kairomone is caused by bacteria (Tollrian, 1993; Tollrian and von Elert, 1994). In fact, Dodson (Dodson, 1988) found in laboratory experiments (no direct sunlight exposure) run at 21°C with unfiltered water that the kairomone ("water-soluble chemical") effect on the behavioral response of *Daphnia* lasted for up to 7 h. This again points to a rapid bacterial degradation, but this needs to be assessed under controlled experimental conditions. Nevertheless, the data from Dodson (Dodson, 1988) and ours suggest that the kairomone of *Chaoborus* has a short half-life.

Another point that deserves attention relates to the observation by Sell (Sell, 2000) on the differential response of *D. pulex* and *D. rosea* to *C. obscuripes* and *C. flavicans* concluding that the former species has a clear response over the latter. The clone of *D. pulex* used in our study originated from a small artificial pond where they cohabitated with four different species of *Chaoborus* (*C. flavicans, C. obscuripes, C. crystallinus*, and *C. pallidus*). Therefore, it would be interesting to test whether a difference in the response of *D. pulex* exists to the kairomone of different *Chaoborus* species. Finally, the role of active versus inactive kairomones in affecting the depth preference of *Daphnia* needs to be experimentally tested.

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