

## Brine shrimp larval photoresponses involved in diel vertical migration: Activation by fish mucus and modified amino sugars

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### Abstract

Photoresponses involved in the descent phase of nocturnal diel vertical migration of brine shrimp (*Artemia franciscana*) naupliar larvae were measured in a laboratory system that mimicked the underwater angular light distribution. The test hypothesis was that kairomones from fish that activate photoresponses involved in DVM include degradation products of polysaccharides in their external mucus. Studies focused on the glycosaminoglycans heparin, chondroitin sulfate A, and hyaluronic acid and their repeating disaccharide units. Active molecules were identified by their activation of the descent photoresponse. Mucus from the mummichog (*Fundulus heteroclitus*) activated photoresponses at concentrations down to  $10^{-5}$  g wet weight of mucus  $L^{-1}$ . Water that had previously contained this species had (1) active molecules that were <10 kDa in size, (2) polysaccharides in the >10-kDa, <30-kDa fraction that could be acted upon by heparinase and chondroitinase to produce active molecules, and (3) enzymes in the >30-kDa, <100-kDa fraction that acted on heparin and chondroitin sulfate polysaccharides to produce active molecules. The repeating disaccharide subunits of heparin, chondroitin sulfate A, and hyaluronic acid-induced photoresponses. The disaccharide subunit of hyaluronic acid was the most potent disaccharide, as it activated photoresponses at concentrations as low as  $10^{-9}$  M. Tests with different subunits found that compounds with the most potent biological activity were disaccharides with either a sulfamino or acetylamino group on carbon 2 of the hexoseamine. Collectively, the results support the hypothesis and indicate that enzymatic degradation products of sulfated and acetylated fish mucus can serve as kairomones.

Nocturnal diel vertical migration (DVM) is commonly observed among zooplankton and is characterized by a single daily ascent with minimum depth reached between sunset and sunrise and maximum depth attained during the day (Forward 1988). The most accepted hypothesis for functional significance of DVM is that zooplankton ascend to feed during times of low light levels near the surface (reviewed by Pearre [1979]) and descend to dim lit areas during the day to avoid visual predators (Zaret and Suffern 1976; Stich and Lampert 1981).

The strength (e.g., Williamson and Magnien 1982; Frost 1988; Ohman 1990; Stirling et al. 1990) and pattern (e.g., Ohman et al. 1981; Neill 1990) of DVM are related to predator abundance. In field (e.g., Dini and Carpenter 1988, 1991; Ringelberg et al. 1991) and laboratory studies (Dawidowicz et al. 1990; Tjossen 1990; Loose 1993), DVM was absent or reduced when predators were absent and well developed in their presence. Rapid changes in DVM in the presence and absence of predators (Bollens and Frost 1989b, 1991; Ringelberg et al. 1991; Neill 1992) indicate that a phenotypic response occurs in which zooplankton behavior and physiology are modified by cues from planktivores. With the exception of one study (Bollens and Frost 1989a), evidence indicates prey use chemical cues (kairomones) to detect predators (e.g., Dodson 1990; Neill 1990, 1992; Ringelberg 1991a,b; Loose 1993; Loose et al. 1993). Accordingly, photoresponses involved in DVM are modified by kairomones from planktivorous fishes (e.g., Ringelberg 1995; Ringelberg and Van Gool 1995; Van Gool and Ringelberg 1995; Van Gool 1998).

### Acknowledgments

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Further characterization of kairomones that alter photoresponses is the topic of the present study using larvae of the brine shrimp *Artemia franciscana*.

Adult brine shrimp undergo nocturnal DVM (Lenz 1980). DVM has not been measured for larval brine shrimp but is inferred because (1) larval photoresponses to relative rate of change in light intensity (Forward and Hettler 1992) are consistent with those for zooplankton that undergo nocturnal DVM (Forward 1988), and (2) larvae are readily preyed upon by visual planktivores such as small fish in the laboratory (Forward and Hettler 1992). The relative rate of increase in light intensity, as occurs at sunrise, induces negative phototaxis in brine shrimp larvae that would lead to a descent response (Forward and Hettler 1992). Step increases in light produce similar descent responses in brine shrimp larvae and have been used to quantify activation of this negative phototaxis by fish kairomones (Forward and Hettler 1992; Forward and Rittschof 1993; McKelvey and Forward 1995). The response is absent if larvae are incubated for 24 h in water free from biologically active molecules but is present after incubation with fish (Forward and Hettler 1992) and incubation in water that had previously contained fish (Forward and Rittschof 1993; McKelvey and Forward 1995). Activation of the response occurs within 5 min of exposure to the kairomones (Forward and Rittschof 1993), and photoresponsiveness increases with increasing concentration of the kairomones (McKelvey and Forward 1995). Active molecules are (1) <10 kDa in size (Forward and Rittschof 1993; McKelvey 1997), (2) stable when exposed to temperature from  $-80$  to  $129^{\circ}C$ , (3) stable upon exposure to pH from 1.5 to 14, (4) hydrophilic, and (5) not ammonia/ammonium (McKelvey 1997).

Thus, the test hypothesis for the present study is that kairomones from fish include degradation products of polysac-

charides in their external mucus. Mucus is composed of protein moieties (5% of dry weight) covalently bonded to polysaccharide chains (95% of dry weight) that are termed glycosaminoglycans (GAGs; Shephard 1994). The chains are composed of repeating disaccharide units, each with a molecular weight of 400–600 Da (Brimacombe and Webber 1964; Fransson 1985), which is within the size range for fish kairomones that activate photoresponses in brine shrimp larvae (Forward and Rittschof 1993; McKelvey 1997). Early studies indicated that GAGs were present in fish mucus (Wasserman et al. 1972; Wold and Selset 1977), but the specific types of GAGs were not identified. In general, the four main types of GAGs are (1) hyaluronic acid, (2) chondroitin sulfate and dermatan sulfate, (3) heparan sulfate and heparin, and (4) keratan sulfate. All types of GAGs, except keratan sulfate, were studied because (1) chondroitin sulfate and hyaluronic acid are reported in fish mucus (van de Winkel et al. 1986; Karamanos et al. 1991), (2) secretory products of fish epithelial mucous cell produce polysaccharides with terminal groups found in chondroitin sulfate (Hidalgo et al. 1987), and (3) preliminary trials indicated that heparin activated brine shrimp larval photoresponses involved in DVM (McKelvey 1997). Keratan sulfate and heparin are not reported in fish mucus.

GAGs are distinguished by the sugar residues, type of linkage between residues, and number and location of sulfated esters, acetylamino, and sulfamino groups (Fransson 1985). The major repeating disaccharide unit of heparin is uronic acid with a sulfate at the 2 carbon position coupled by an  $\alpha$ 1–4 glycoside linkage to D-glucosamine having a sulfate ester on carbon 6 and a sulfamino group on carbon 2 (Fig. 1). The sulfamino group is unique to heparin and heparan sulfate. The major disaccharide in chondroitin sulfate A (Fig. 1) is uronic acid coupled by an  $\alpha$ 1–3 glycoside linkage to D-galactose amine that has a sulfated ester on carbon 4 and an acetylamino on carbon 2. The disaccharide of hyaluronic acid is similar, having uronic acid coupled by a  $\beta$ 1–3 glycoside linkage to D-glucosamine with an acetylamino on carbon 2 (Fig. 1). Hyaluronic acid lacks sulfates.

The components of fish mucus that activate photoresponses involved in DVM of larval brine shrimp were characterized by testing (1) fish mucus; (2) products from digestion of fish mucus with heparinase and chondroitinase; (3) heparin, chondroitin sulfate, and hyaluronic acid disaccharides; and (4) subunits of these disaccharides. The results support the hypothesis that the breakdown products of mucopolysaccharides function as fish kairomones.

## Materials and methods

*General procedures*—*A. franciscana* Kellog (Hontoria and Amat 1992) naupliar larvae were hatched from cysts obtained from the Great Salt Lake (Sanders Brine Shrimp). During hatching, cysts were incubated in 100-kDa filtered seawater (clean seawater) having a salinity of 31 psu and temperature of 23°C and continuously illuminated with white light from an incandescent lamp. Clean seawater was prepared by septic filtration of water from the Newport River Estuary (North Carolina) to remove molecules larger than

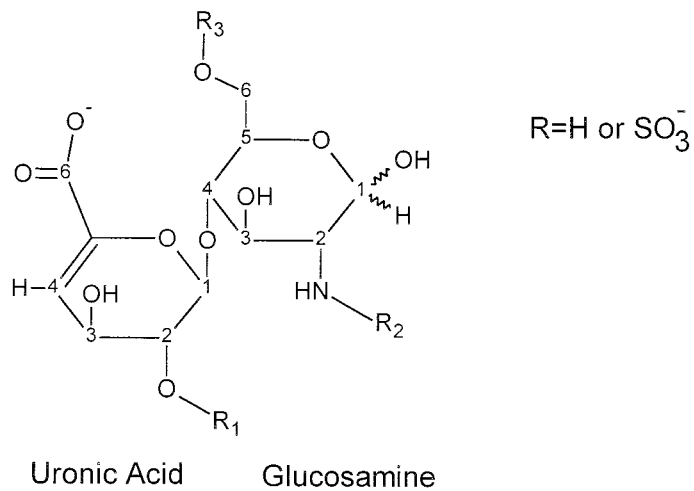
100 kDa and aging for a minimum of 1 week. This procedure removed biologically active molecules and produced water with a consistent chemical composition (e.g., Rittschof et al. 1983). This water does not contain chemical cues that activate larval photoresponses involved in DVM (Forward and Hettler 1992; Forward and Rittschof 1993; McKelvey and Forward 1995) and was used to make up the test solutions.

Several hours after hatching, nauplii were transferred into a 500-ml solution containing *Dunaliella tertiolecta* Grand, 1967 (concentration about  $10^6$  cells  $L^{-1}$ ; 23°C) and allowed to feed for 5 h under continuous white light (cool white fluorescent lamps, intensity =  $1.8 \times 10^{15}$  photons  $m^{-2} s^{-1}$ ). Nauplii were then filtered from the solution, washed twice with clean seawater, placed in aerated clean seawater, and starved until experimentation about 18 h later. During this time they were light adapted to the above fluorescent lamps. This pretreatment enhanced sensitivity to predator odors (Forward and Hettler 1992). After pretreatment, nauplii were exposed for a minimum of 1 h to test chemicals, after which photoresponses were tested. Activation of photoresponses by chemical cues from fish occurs within about 5 min and becomes maximal within an hour of exposure (Forward and Rittschof 1993).

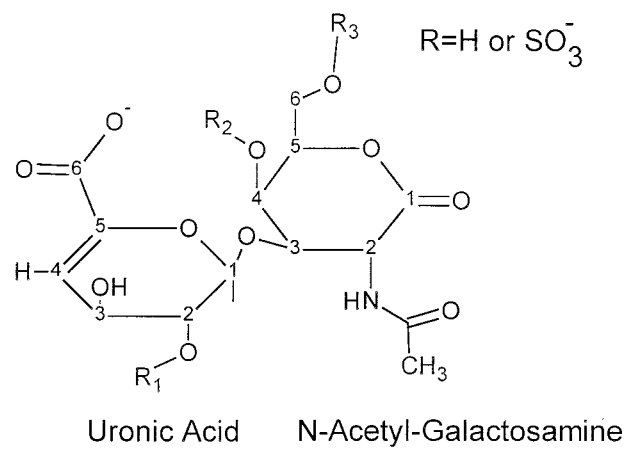
Photoresponses were tested in a room maintained at 23°C in an apparatus designed to produce a light field similar to that occurring underwater during the day (see Forward et al. [1984] for a detailed description). The light stimulus source was a 300-W incandescent lamp filtered to the blue-green region with a Corning no. 4-96 filter (Kopp Glass). The transmitted wavelengths encompass the major spectral sensitivity maxima of adult brine shrimp (Aiken and Hailman 1978; Bradley and Forward 1984). Light intensity was increased in steps by removing fixed neutral-density filters. The actual intensities were measured with a radiometer (E, G, and G; model 550). During experimentation, nauplii were illuminated with far-red light (maximum transmission 775 nm) and observed with a video system (Forward 1985). Adult brine shrimp are insensitive to light in this wavelength region (Bradley and Forward 1984), and prior tests indicated that nauplii were unresponsive to this light (e.g., Forward and Hettler 1992).

The general photostimulation procedure was to adapt nauplii to light ( $1.5 \times 10^{15}$  photons  $m^{-2} s^{-1}$ ) for at least 15 min. After adaptation, the light intensity was increased by removing fixed neutral-density filters for 3 s. The filters were returned and 75 s later nauplii were stimulated with the next greater increase. A previous study (Forward and Hettler 1992) found that nauplii returned to their unstimulated swimming pattern within the 75-s interval, and photoresponses remained constant upon repetitive stimulation. Behavioral responses and time (field/frame counter; QSI Systems) were recorded on videotape. Each test group of nauplii (density = about  $14 ml^{-1}$ ) received eight light stimuli that ranged from a 9 to 80% increase over the initial level. Past experiments (Forward and Hettler 1992; Forward and Rittschof 1993) found that if a response was not present by an 80% increase, it did not occur at larger increases. Stimuli were presented in order from the least to greatest percent increase because prior stimulation did not affect subsequent responses (Forward and Hettler 1992). For all experiments, groups of

## Heparin Disaccharides



## Chondroitin Disaccharides



## Hyaluronic Acid Disaccharide

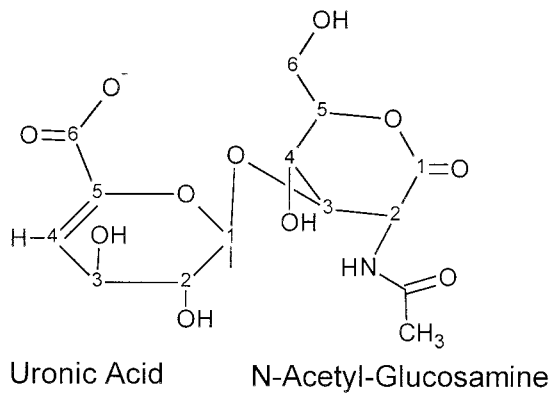


Fig. 1. Heparin, chondroitin sulfate A, and hyaluronic acid disaccharides with positions (R) for sulfated esters and hydrogen groups. The carbons on each ring are numbered 1-6. The different R groups are also numbered (e.g., R1) for reference in the text.

nauplii were tested only once at each stimulus condition, and each experiment was replicated five times. To reduce the possible influence of a biological rhythm in photoresponses, all experiments were conducted between 1200 and 1600 h.

For analysis of directional movement during each replicate, the swimming direction of at least 25 haphazardly selected nauplii was measured at 10° intervals. A descent response was defined as movement directly down ( $\pm 10^\circ$ ). Control movements were measured about 10 s before the onset of each stimulus and oriented responses measured 1 s after stimulation. A previous study found that a descent response, if present, was well developed by this time (Forward and Hettler 1992).

The percentage of larvae descending was calculated for each trial at each stimulus condition. Means, standard deviations (SDs), and standard errors (SEs) for combined trials were calculated after data were arcsin transformed. Back-transformed means and standard errors are plotted on the figures. For each chemical stimulus, relative changes in the percentage of larvae descending before and after light stimulation were compared using a Friedman's test (Zar 1984). A one-tailed statistical test was conducted, as light stimulation was expected to increase the percent descending. If the Friedman's test indicated a significant difference among light stimulation levels, the smallest percent increase in light intensity to evoke a significant response (threshold) was determined by comparing relative changes in percent descending to control values using a Wilcoxon paired-sample test (one tailed) at an experimental-wise error rate of 0.05.

**Test chemicals**—Experiments were designed to test naupliar photoresponses following exposure to different concentrations of specific test chemicals. Greater activation of photoresponses involved in DVM was indicated by a reduction in the minimum percent increase in light intensity (threshold) that evoked a significant descent response. The four groups of test chemicals were (1) fish mucus, (2) size-fractionated and enzyme-treated size-fractionated components of predator odor water, (3) commercially purified heparin, chondroitin sulfate A, and hyaluronic acid disaccharides (Sigma), and (4) commercially purified disaccharide subunits (Sigma). Fish incubation levels and test concentrations were based on previous studies (Forward and Rittschof 1993; McKelvey and Forward 1995; McKelvey 1997).

**Fish mucus**—Fish mucus was collected from mummichogs (*Fundulus heteroclitus*) because a previous study found that naupliar photoresponses were activated by exposure to water that had previously contained this species (McKelvey and Forward 1995). The solution of fish mucus was prepared from five large female fish (average weight = 14.6 g). Each fish was removed from clean seawater with a small net and placed damp and dorsal-side-up in a large finger bowl (19 cm diameter). With a latex surgical gloved hand, the wiper lightly grasped the head of the fish over the opercula. Using the other similarly gloved hand, the wiper gently stroked the dorsal surface of the fish three times using a preweighed Kimwipe®. Fish were exposed to air for <30 s during the procedure and returned immediately to water. They showed no sign of disease and were healthy in ap-

pearance for the next week. The five wipes carrying the mucus from five fish were weighed again, and the amount of material removed from the surface of the fish determined by the difference between initial and final weights. The wipes were then placed in a 100-ml beaker, to which 50 ml of clean seawater was added. After covering, the beaker was incubated at 23°C on an orbital shaker at 60 rpm for 40 min. At the end of the incubation interval, 42.5 ml of liquid was recovered by decanting liquid from the beaker and squeezing the Kimwipes® with a latex surgical gloved hand. This solution was frozen until use and had a sulfated sugar concentration of 260  $\mu\text{g ml}^{-1}$  as determined using heparin disaccharide (Sigma H-9267) as a standard in the phenol sulfuric procedure (Ashwell 1955). For tests with fish mucus, nauplii were incubated in 600 ml of clean seawater having a defined wet weight concentration ( $\text{g wet weight of fish mucus L}^{-1} = \text{g L}^{-1}$ ). The control solution was generated by repeating the above procedure with clean Kimwipes® and was tested at the highest equivalent fish mucus concentration (0.1  $\text{g L}^{-1}$ ).

**Size-fractionated components of predator odor water**—Previous studies found that water that had previously contained fish had chemical cues that activated naupliar photoresponses (Forward and Rittschof 1994; McKelvey and Forward 1995). It was hypothesized that some of these chemical cues originated from fish mucus. Thus, the size fractionation procedure was to incubate *F. heteroclitus* for 5 min at a ratio of 330  $\times$  g of fish to 1 liter of clean seawater to produce predator odor water. The fish were then removed, the water cooled immediately, and subjected to cascade pressure dialysis (Amicon). The three size classes of molecules created by dialysis were (1) >30 kDa, <100 kDa; (2) >10 kDa, <30 kDa; and (3) <30 kDa. Volumes of each size fraction were adjusted to a constant level by the addition of nanopure water. Dilutions of these fractions were made up in clean seawater for bioassay.

The incubation procedure was based on the fish weight-to-water volume ratio that produced active molecules after a 24-h period in a previous study (McKelvey 1997). The short (5 min) incubation period was used to collect enzymes and polysaccharides that are associated with fish without collected artificial breakdown products resulting from the build-up of metabolites and bacteria in the incubation water.

First, to reconfirm that the active molecules were <10 kDa in size (Forward and Rittschof 1993; McKelvey and Forward 1995), naupliar photoresponses were tested upon exposure to the <30-kDa fraction (concentrations 1:25, 1:50, 1:100 dilution of original solution) and >10-kDa, <30-kDa fraction (concentration 1:25 dilution of original solution) and >30 kDa <100 kDa (concentration 1:25 dilution of original solution). The second experimental series determined whether biological activity could be generated from the inactive >10-kDa to <30-kDa fraction by incubation with chondroitinase ABC lyase and heparinase lyase. Tests with hyaluronidase were performed previously by McKelvey (1997). Chondroitinase ABC lyase digests sulfated polysaccharides and generates disaccharides with  $\alpha$ 1-3 glycoside linkages, as found in the chondroitin sulfate A disaccharide. Heparinase lyase digests sulfated polysaccharides and gen-

erates disaccharides with  $\alpha$ 1–4 glycoside linkages, as found in the heparin disaccharide. The >10-kDa, <30-kDa solution was divided in three equal aliquots that were subjected to different treatments for 1 h at 23°C. One aliquot received no added enzymes and served as a control. The next aliquot was incubated with 10 units of chondroitinase ABC lyase (Sigma C-2905) and the final aliquot was incubated with 100 units of heparinase lyase (Sigma H-2519). Following incubation, all aliquots were filtered through a 30-kDa membrane to remove the enzymes and the filtrate frozen. After thawing, a series of dilutions (1:25 to 1:200 dilution of original solution) of each filtrate was made up in clean seawater and tested for activation of photoresponses. Possible additional control experiments were to test whether the enzymes themselves and/or impurities in the enzyme preparation had active molecules. These controls were not performed because McKelvey (1997) found that the addition of either heparinase or hyaluronidase to clean seawater did not generate active molecules within 8 h.

The third experimental series was designed to determine whether there were chondroitinase ABC lyase-like and heparinase lyase-like enzymes in the predator odor water. This was accomplished by adding purified polymer substrates to size-fractionated predator odor water and determining if biologically active molecules were produced. The >30-kDa, <100-kDa fraction was divided into three equal aliquots (2.5 ml each), all of which were incubated under different conditions at 23°C for 20 h. The first aliquot served as an incubation control and received no additional substrate. The second aliquot was combined with 600 units of heparin polysaccharide (Elkins-Sinn) and the third aliquot combined with 86 mg of chondroitin sulfate A polysaccharide (Sigma C-8529). The repeating disaccharide units of heparin polysaccharide have sulfated esters at the R1, R2, and R3 locations in Fig. 1, whereas the chondroitin sulfate A disaccharide has a sulfated ester at R2 and hydrogen at R1 and R3 (Fig. 1). After incubation each solution was frozen. When testing for photoresponse activation, 20% of each aliquot was added to 600 ml of clean water. Additional control solutions had separate additions of equivalent incubation amounts of heparin and chondroitin sulfate A to clean seawater. These controls were performed to insure that the observed responses did not result from the levels of chondroitin sulfate A and heparin polysaccharides in the solutions but rather were due to products generated by enzymatic breakdown of the polysaccharides.

*Components structurally related to heparin subunits*—Subunits of heparin were tested to determine the structural site(s) that activate naupliar photoresponses. All solutions were made up in 600 ml of clean seawater on the test day. The first experiment tested the heparin disaccharide (R1, R2, R3 = SO<sup>3-</sup>; Sigma H-9267; Fig. 1) at concentrations of  $5 \times 10^{-8}$ – $10^{-6}$  M. Heparin disaccharide is  $\alpha$ - $\Delta$ UA-2S-[1 $\rightarrow$ 4]-GlcNS-6S where  $\Delta$ UA = 4-deoxy-L-threo-hex-4-enopyranosyluronic acid, GlcN = D-glucosamine, NS = N sulfo, 2S = 2-sulfate, and 6S = 6-sulfate. This disaccharide would be produced by the action of heparinase lyase on the heparin polysaccharide. The unique part of this molecule is the sulfamino group on the second carbon (signature sulfamino

group: R2; Fig. 1) of glucosamine. Thus, the second experiment tested the heparin disaccharide that had only this sulfamino group ( $\alpha$ - $\Delta$ UA-[1 $\rightarrow$ 4]-GlcNS; R1 and R3 = H, R2 = SO<sup>3-</sup>; Sigma H-1145) at concentrations between  $10^{-8}$  to  $10^{-6}$  M. Because both experiments found that photoresponse threshold decreased as the concentration increased, subsequent experiments tested subunits at a single concentration ( $5 \times 10^{-7}$  M) that evoked a very strong response in the first two experiments. Isolated trials indicated that if photoresponse activation did not occur at this concentration, it was absent at higher concentrations. Subsequent experiments tested (1) heparin disaccharide without any sulfates ( $\alpha$ - $\Delta$ UA-[1 $\rightarrow$ 4]-GlcN; R1, R2 and R3 = H; Fig. 1; Sigma H-9276), (2) heparin disaccharide with all sulfated esters but lacks the signature sulfamino moiety ( $\alpha$ - $\Delta$ UA-2S-[1 $\rightarrow$ 4]-GlcN-6S; R1 = SO<sup>3-</sup>, R2 = H, R3 = SO<sup>3-</sup>; Fig. 1; Sigma H-8892), (3) heparin disaccharide with only the two sulfates on glucosamine ( $\alpha$ - $\Delta$ UA-[1 $\rightarrow$ 4]-GlcNS-6S; R1 = H, R2 = SO<sup>3-</sup>, R3 = SO<sup>3-</sup>; Fig. 1; Sigma H-1020), (4) heparin disaccharide with the sulfate at carbon 2 of uronic acid and only the sulfamino group at carbon 2 of glucosamine ( $\alpha$ - $\Delta$ UA-2S-[1 $\rightarrow$ 4]-GlcNS; R1 = SO<sup>3-</sup>, R2 = SO<sup>3-</sup>, R3 = H; Fig. 1; Sigma H-9392), (5) glucosamine with a sulfamino group on carbon 2 and sulfated ester at carbon 6 (D-glucosamine 2,6-disulfate; R2 = SO<sup>3-</sup>, R3 = SO<sup>3-</sup>; Fig. 1; Sigma G-7514), (6) glucosamine with only the sulfamino group at carbon 2 (D-glucosamine 2-sulfate; R2 = SO<sup>3-</sup>, R3 = H; Fig. 1; Sigma G-7889), (7) glucosamine with only a sulfate at the 6 carbon position (D-glucosamine 6-sulfate; R2 = H, R3 = SO<sup>3-</sup>; Fig. 1; Sigma G-8641), (8) D-glucosamine alone (R2 = H, R3 = H; Fig. 1; Sigma G-4875) with no sulfates, (9) D-glucuronic acid alone (R1 = H; Fig. 1; Sigma 8645), and (10) D-glucose (Sigma G-8270). Glucuronic acid was considered structurally equivalent to uronic acid.

*Components structurally related to chondroitin sulfate subunits*—The repeating disaccharide in chondroitin sulfate A polysaccharide is sulfated on carbon 4 of galactose ( $\alpha$ - $\Delta$ UA[1 $\rightarrow$ 3]-GalNAc-4S; R1 and R3 = H, R2 = SO<sup>3-</sup>; Fig. 1; Sigma C4045). This disaccharide differs from heparin (above) by having a 1–3 disaccharide linkage and by the substitution of N-acetyl-D-galactosamine for N-sulfamino-D-glucosamine. It was tested at concentrations of  $10^{-9}$  to  $5 \times 10^{-7}$  M to determine the relationship between photoresponse threshold and chondroitin sulfate A disaccharide concentration. Activities of other chondroitin di- and monosaccharides were tested at a single concentration of  $5 \times 10^{-7}$  M for comparison with the heparin results. All chondroitin disaccharides had the acetyl amino group on galactose and different combinations of sulfated esters including ones with (1) a single sulfate on carbon 6 of galactosamine ( $\alpha$ - $\Delta$ UA-[1 $\rightarrow$ 3]-GalNAc-6S; R1 = H, R2 = SO<sup>3-</sup>, R3 = H; Fig. 1; Sigma C4170), (2) double ester sulfates at carbon 2 on uronic acid and carbon 4 on galactosamine ( $\alpha$ - $\Delta$ UA-2S-[1 $\rightarrow$ 3]-GalNAc-4S; R1 = SO<sup>3-</sup>, R2 = SO<sup>3-</sup>, R3 = H; Fig. 1; Sigma C3670), and (3) no sulfates ( $\alpha$ - $\Delta$ UA-[1 $\rightarrow$ 3]-GalNAc; R1, R2, and R3 = H; Fig. 1; Sigma C3920).

The monosaccharides of N-acetyl galactosamine that were tested with different sulfate esters included ones with (1) sulfate at carbons 4 and 6 (N-acetyl-D-galactosamine 4–6

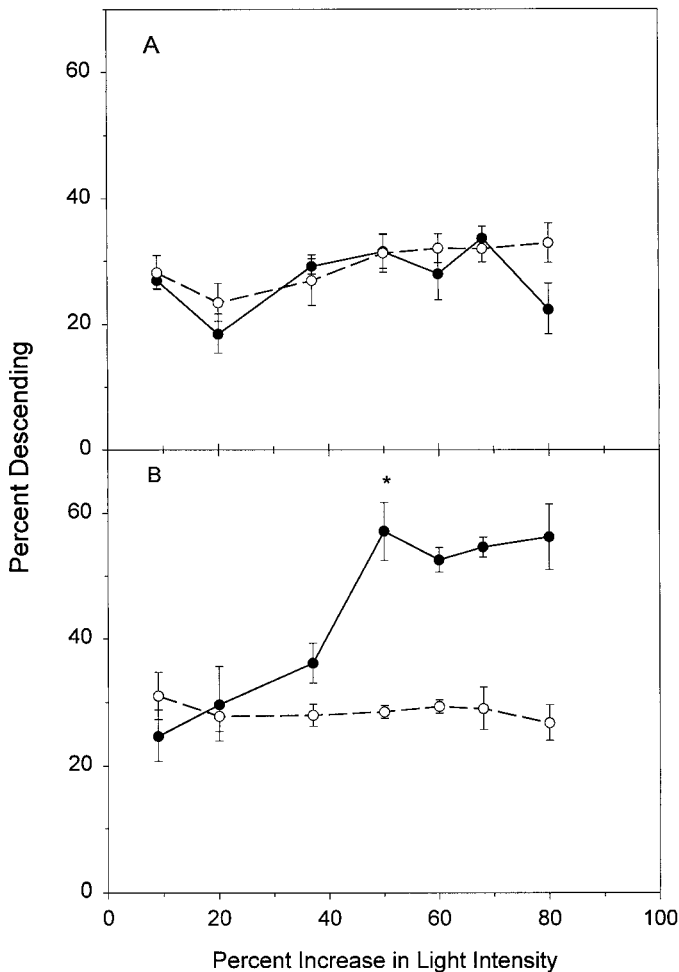


Fig. 2. Percentage of brine shrimp nauplii showing a descent response upon step (percent) increases in light intensity when exposed to clean seawater (A) and 1:50 dilution of the <30-kDa molecular size fraction of predator odor water (B). The solid line (solid circles) shows responses upon light stimulation, while the dashed line (open circles) shows the percent descending prior to stimulation (control). An asterisk indicates the smallest percent increase in intensity that evokes a descent response significantly ( $P < 0.05$ ; Wilcoxon pair-sample test) greater than the control level (threshold). The sample size was five. Means and standard errors are plotted.

disulfate; R2 and R3 =  $\text{SO}_3^-$ ; Fig. 1; Sigma A6051), (2) a sulfate at carbon 4 (*N*-acetyl-D-galactosamine 4-sulfate; R2 =  $\text{SO}_3^-$ , R3 = H; Fig. 1; Sigma A5926), and (3) no sulfates (*N*-acetyl-D-galactosamine; R1 and R2 = H; Fig. 1; Sigma A2795). Other galactosamine monosaccharides lacked the acetyl group but had (1) a sulfate at carbon 6 (D-galactosamine 6-sulfate; R2 = H; R3 =  $\text{SO}_3^-$ ; Fig. 1; Sigma G2022) and no sulfates (D-galactosamine; R2 and R3 = H; Fig. 1; Sigma G0264). In addition, the ring without the amino group (D-galactose; Sigma G6404) was tested.

*Components structurally related to hyaluronic acid subunits*—The repeating disaccharide subunit of hyaluronic acid consists of uronic acid coupled by a 1–3 linkage to *N*-acetylglucosamine ( $\alpha$ - $\Delta$ UA-[1→3]-GlcNAc; Sigma H9649).

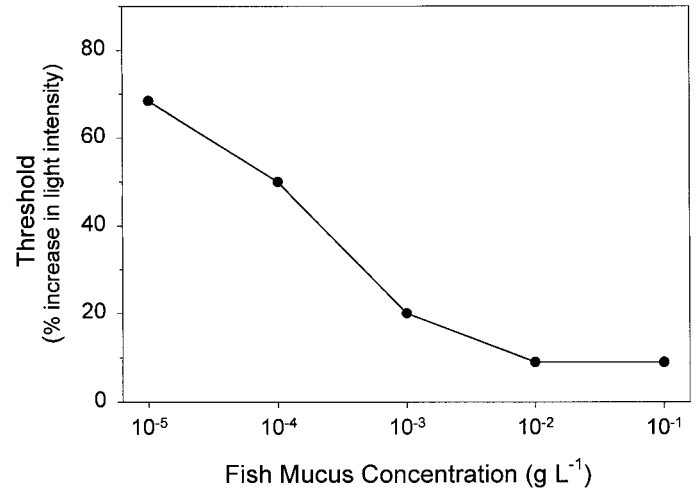


Fig. 3. The minimum percent increase in light intensity that evoked a descent response that was significantly different from the control (threshold) upon exposure to different concentrations (g wet weight L<sup>-1</sup>) of fish mucus. The sample size was five for determining each threshold.

This molecule was tested at concentrations of  $5 \times 10^{-10}$  to  $10^{-6}$  M. The previously untested monosaccharide constituent of this disaccharide *N*-acetyl-D-glucosamine (Sigma A8625) was tested at a single concentration of  $5 \times 10^{-7}$  M.

## Results

*Fish mucus*—When tested in clean seawater, nauplii never showed a significant descent response upon an increase in light intensity (Fig. 2A). In contrast, when exposed to fish mucus and molecular size fractions that had previously contained fish, nauplii displayed the descent photoresponse. A representative response pattern is shown in Fig. 2B for nauplii exposed to the <30-kDa fraction of predator odor water (1:50 dilution of the original solution). The mean percentage of nauplii descending before light stimulation remained relatively constant. In contrast, the percentage of nauplii descending upon light stimulation increased to a maximum level as the percent increase in light intensity became greater. These relationships were constructed for each test condition and photoresponses quantified by determining the smallest increase in light intensity that induced a significant descent response (threshold).

As the concentration of fish mucus increased the threshold decreased (Fig. 3). A significant response was not observed at fish mucus concentrations below  $10^{-5}$  g L<sup>-1</sup>, such as  $10^{-6}$  g L<sup>-1</sup>. The control for these experiments was exposure to Kimwipe® water that did not evoke a significant response at a concentration equivalent to 0.1 g of fish mucus L<sup>-1</sup>. The interpretation of Fig. 3 is that as the concentration of active molecules increased naupliar sensitivity to increases in light intensity increased as indicated by the decrease in the threshold. Thus, in subsequent experiments the threshold was determined and used to gauge the relative activity level of test molecules.

Predator odor water was assumed to contain fish mucus

Table 1. Responses to different size fractions in predator odor water. Dilution is the ratio of volume of size-fractionated material to volume of clean seawater. Threshold is the lowest percent increase in light intensity that induced a significant descent photoresponse. NR (no response) indicates none of the test increases in light intensity induced a significant response.

Size fraction	Dilution	Threshold (%)
>30 kDa, <100 kDa	1 : 25	NR
>10 kDa, <30 kDa	1 : 25	NR
<30 kDa	1 : 25	9
	1 : 50	50
	1 : 100	NR

and its breakdown products. If this water was size fractionated, the >30-kDa, <100-kDa fraction and the >10-kDa, <30-kDa fractions did not contain active molecules at the highest test concentration (1 : 25 dilution of original solution; Table 1). In contrast, the <30-kDa fraction evoked a maximum response at 1 : 25 dilution of original solution (threshold = 9% light intensity increase) and a significant response at 1 : 50 dilution (Table 1; Fig. 2). These results are consistent with the interpretation that the active molecules have molecular weights of <10 kDa.

The biologically inactive >10-kDa, <30-kDa fraction was divided and treated with heparinase lyase and chondroitinase ABC lyase to determine if the mixture contained heparin-like and chondroitin sulfate-like substrates that, when digested by the enzyme, would yield biologically active products. After incubation the enzyme was removed by ultrafiltration through a 30-kDa membrane and the filtrate tested for activity. The control for these experiments was to expose the >10-kDa, <30-kDa fraction for the same length of time (1 h) to the temperature (23°C) used for the enzyme incubations. At a 1 : 25 dilution of the original solution, the control solution did not induce a significant photoresponse (Table 2). Exposure to heparinase lyase induced significant responses at concentrations from 1 : 25 to 1 : 100 but not at 1 : 200 dilution of original solution. Similarly, after exposure to chondroitinase ABC lyase, the mixture induced a maximum response at a dilution of 1 : 25 of the original solution. Thus, the >10-kDa, <30-kDa fraction contained polysaccharides that were digested by both heparinase and chondroitinase ABC lyase to yield biologically active products.

To determine whether the >30-kDa, <100-kDa fraction contained enzymes with heparinase lyase and chondroitinase ABC lyase activity, this fraction was separately incubated with polysaccharide substrates of heparin and chondroitin sulfate A. Subsequent activity could be due to (1) the presence of heparin and chondroitin sulfate A polysaccharides and/or (2) the production of small molecules due to enzymatic action. The >30-kDa, <100-kDa fraction did not contain active molecules (control; Table 3). If the incubation concentration of heparin polysaccharide was added to clean seawater, photoresponses were activated with a threshold of 80% (heparin control; Table 3). However, after incubation of the >30-kDa, <100-kDa fraction with heparin, the resulting mixture contained an increased concentration of active molecules because the photoresponse threshold was reduced to

Table 2. Treatment of >10 kDa, <30 kDa fraction of fish water with heparinase and chondroitinase ABC lyase. The control was an untreated aliquot of this fraction. Symbols are as described for Table 1.

Treatment	Dilution	Threshold (%)
Control	1 : 25	NR
Heparinase lyase	1 : 25	9
	1 : 50	20
	1 : 100	37
	1 : 200	NR
Chondroitinase ABC lyase	1 : 25	9

20% (heparin in >30 kDa, <100 kDa; Table 3). Thus, there was heparinase lyase-like enzymatic activity in the >30-kDa, <100-kDa fraction.

The results with chondroitin sulfate A were simpler to interpret. The addition of the incubation level of chondroitin sulfate A polysaccharide to clean seawater did not activate photoresponses (chondroitin sulfate A control; Table 3). However, incubation of this polysaccharide with the >30-kDa, <100-kDa fraction produced active molecules that induced a photoresponse with a threshold of 37% (chondroitin sulfate A in >30 kDa, <100 kDa; Table 3). Thus, there was chondroitinase lyase-like enzymatic activity in the >30-kDa, <100-kDa fraction.

*Components structurally related to heparin subunits*—The increase in activity after incubation of heparin with the >30-kDa, <100-kDa fraction (Table 3) could be due to the production of the heparin disaccharide with sulfated esters at three locations (R1, R2, R3; Fig. 1) of the molecule ( $\alpha$ - $\Delta$ UUA-2S-[1 $\rightarrow$ 4]-GlcNS-6S). The addition of this disaccharide to clean seawater activated photoresponses and produced a concentration/response relationship (Fig. 4, solid line) that is similar in shape to that for fish mucus (Fig. 3). The photoresponse threshold increased as the concentration decreased, with  $10^{-7}$  M as the lowest active concentration. Tests with lower concentrations (e.g.,  $5 \times 10^{-8}$  M) failed to activate photoresponses.

A distinctive feature of heparin disaccharides is the signature sulfamino moiety at the 2 carbon location (R1 = H, R2 =  $\text{SO}_3^-$ , R3 = H; Fig. 1). The heparin disaccharide with only this signature sulfamino group ( $\alpha$ - $\Delta$ UUA-[1 $\rightarrow$ 4]-GlcNS)

Table 3. Responses after the addition of heparin or chondroitin sulfate A polysaccharide to the >30 kDa, <100 kDa fraction of fish water. Controls included testing this fraction without treatment (control) and adding an equivalent amount of either heparin (heparin control) or chondroitin sulfate A polysaccharide (chondroitin sulfate control) to clean seawater. Symbols are as in Table 1.

Treatment	Threshold (%)
Control (untreated >30-kDa, <100-kDa fraction)	NR
Heparin control	80
Heparin in >30-kDa, <100-kDa fraction	20
Chondroitin sulfate A control	NR
Chondroitin sulfate A in >30-kDa, <100-kDa fraction	37

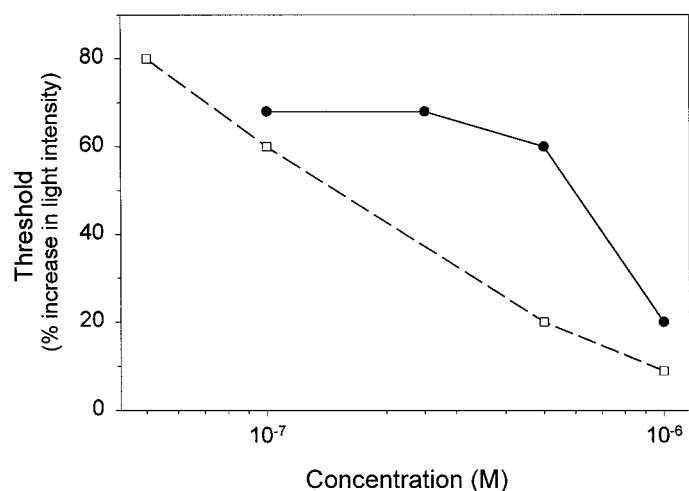


Fig. 4. The minimum percent increase in light intensity that evoked a descent response that was significantly different from the control (threshold) upon exposure to different molar concentrations of the heparin disaccharide ( $\alpha$ - $\Delta$ UA-2S-[1 $\rightarrow$ 4]-GlcNS-6S; solid line) and heparin disaccharide that lacked all sulfated esters but has the sulfamino group on the second carbon of glucosamine ( $\alpha$ - $\Delta$ UA-[1 $\rightarrow$ 4]-GlcNS; dashed line). The sample size was five for determining each threshold.

was more potent than the parent molecule, as the lowest concentration to evoke a significant photoresponse was  $5 \times 10^{-8}$  M (Fig. 4, dashed line). Lower concentrations (e.g.,  $10^{-8}$  M) failed to activate a photoresponse. Because both the heparin disaccharide with all ester sulfates and only the signature sulfamino group activated photoresponses, activity was further evaluated by testing different structural subunits at a single concentration of  $5 \times 10^{-7}$  M. This concentration was tested because it induced strong but not maximum photoresponses with both of the above heparin disaccharides (Fig. 4).

Heparin disaccharides that (1) lacked all sulfated esters and (2) had all sulfates except the signature sulfamino group

did not activate photoresponses (1, 2 in Table 4). In contrast, all heparin disaccharides with any combination of sulfates that included the sulfamino group (R2) induced photoresponses (3–6; Table 4). These results indicate that activity of the disaccharide was imparted by the signature sulfamino group on carbon 2.

Considering monosaccharides, glucosamine with a sulfate at the 6 carbon position and sulfamino group at the 2 carbon position (7 in Table 4) and just the sulfamino on carbon 2 (8 in Table 4) were both active. Activity was lost if there was only the sulfate on carbon 6 (9 in Table 4), or with the ring alone as D-glucose (12 in Table 5). Glucosamine (10 in Table 4) and D-glucuronic acid (11 in Table 4) had weak activity as indicated by the high threshold. These results were consistent with the above conclusion that biological activity is highest with the presence of the signature sulfamino group.

*Components structurally related to chondroitin sulfate subunits*—Chondroitin sulfate polysaccharides are composed of repeating disaccharide groups in which uronic acid is connected to galactosamine by a 1–3 glycoside linkage. The major distinguishing characteristic is the *N*-acetyl group on carbon 2 of the galactosamine (Fig. 1). Sulfated esters can occur at locations R1, R2, and R3 (Fig. 1). The repeating disaccharide in chondroitin sulfate A polysaccharide (substrate, Table 3) has a sulfate on carbon 4 of galactosamine ( $\alpha$ - $\Delta$ UA-[1 $\rightarrow$ 3]-GalNAc-4S; Fig. 1). Tests with different concentrations of this disaccharide (Fig. 5) found that the threshold increased as the concentration decreased. Concentrations below  $5 \times 10^{-9}$  M did not induce a significant photoresponse (e.g.,  $10^{-9}$  M). Other disaccharides with different sulfated esters were tested at a single concentration of  $5 \times 10^{-7}$  M (Table 5). Because disaccharides with different sulfate arrangements (1–3; Table 5) and no sulfates (4; Table 5) all induced a maximum photoresponse, the sulfated esters are unnecessary for activity of the disaccharides. All of the monosaccharides of *N*-acetyl-galactosamine with (5, 6; Table 5) and without sulfates (7; Table 5) were equally active with

Table 4. Photoresponse thresholds after exposure to compounds structurally related to heparin subunits at a concentration of  $5 \times 10^{-7}$  M. The R groups are shown in Fig. 1. Dashes occur in R group columns for monosaccharides that are lacking the saccharide moiety that could be substituted. Symbols are as in Table 1.

	R1	R2	R3	Threshold (%)
<b>Disaccharides</b>				
(1) $\alpha$ - $\Delta$ UA[1 $\rightarrow$ 4]-GlcN	H	H	H	NR
(2) $\alpha$ - $\Delta$ UA-2S-[1 $\rightarrow$ 4]-GlcN-6S	SO <sup>3-</sup>	H	SO <sup>3-</sup>	NR
(3) $\alpha$ - $\Delta$ UA-2S-[1 $\rightarrow$ 4]-GlcNS-6S	SO <sup>3-</sup>	SO <sup>3-</sup>	SO <sup>3-</sup>	60
(4) $\alpha$ - $\Delta$ UA-[1 $\rightarrow$ 4]-GlcNS-6S	H	SO <sup>3-</sup>	SO <sup>3-</sup>	20
(5) $\alpha$ - $\Delta$ UA-2S-[1 $\rightarrow$ 4]-GlcNS	SO <sup>3-</sup>	SO <sup>3-</sup>	H	20
(6) $\alpha$ - $\Delta$ UA-[1 $\rightarrow$ 4]-GlcNS	H	SO <sup>3-</sup>	H	20
<b>Monosaccharides</b>				
(7) D-Glucosamine 2,6-disulfate	—	SO <sup>3-</sup>	SO <sup>3-</sup>	20
(8) D-Glucosamine 2-sulfate	—	SO <sup>3-</sup>	H	20
(9) D-Glucosamine 6-sulfate	—	H	SO <sup>3-</sup>	NR
(10) D-Glucosamine	—	H	H	60
(11) D-Glucuronic acid	H	—	—	50
(12) D-Glucose	—	H	H	NR

Table 5. Photoresponses after exposure to components structurally related to chondroitin sulfate subunits at a concentration of  $5 \times 10^{-7}$  M. The positions of the R groups are shown in Fig. 1. Dashes occur in R group columns for monosaccharides that are lacking the saccharide moiety that could be substituted. Symbols are as in Table 1.

Molecule	R1	R2	R3	Threshold (%)
Chondroitin disaccharide				
(1) $\alpha$ - $\Delta$ UA-[1 $\rightarrow$ 3]-GalNAc-4S	H	SO <sup>3-</sup>	H	9
(2) $\alpha$ - $\Delta$ UA-[1 $\rightarrow$ 3]-GalNAc-6S	H	H	SO <sup>3-</sup>	9
(3) $\alpha$ - $\Delta$ UA-2S-[1 $\rightarrow$ 3]-GalNAc-4S	SO <sup>3-</sup>	H	SO <sup>3-</sup>	9
(4) $\alpha$ - $\Delta$ UA-[1 $\rightarrow$ 3]-GalNAc	H	H	H	9
Monosaccharides				
(5) <i>N</i> -acetyl D-Galactosamine 4,6-disulfate	—	SO <sup>3-</sup>	SO <sup>3-</sup>	60
(6) <i>N</i> -acetyl D-Galactosamine 4-sulfate	—	SO <sup>3-</sup>	H	60
(7) <i>N</i> -acetyl D-Galactosamine	—	H	H	60
(8) D-Galactosamine 6-sulfate	—	H	SO <sup>3-</sup>	NR
(9) D-Galactosamine	—	H	H	NR
(10) D-Galactose	—	H	H	NR

a threshold of 60%. Because activity was lost when the acetyl group was absent from galactosamine (8, 9; Table 5) and with D-galactose alone (10; Table 5), the *N*-acetyl group on galactosamine is essential for activity.

*Components structurally related to hyaluronic acid subunits*—The disaccharide of hyaluronic acid was the most active molecule tested, as it induced a significant descent photoresponse at concentrations of  $10^{-9}$  M and higher (Fig. 6). Tests at lower concentrations (e.g.,  $5 \times 10^{-10}$  M) failed to induce a significant response. The subunit of this disaccharide (*N*-acetyl-D-glucosamine) was also active, because it had a threshold of 60% when tested at a concentration of  $5 \times 10^{-7}$  M. It should be noted that this was the same response observed for *N*-acetyl-D-galactosamine at the same concentration (Table 5). Results with other possible monosaccharides (D-glucosamine-6-sulfate; D-glucosamine) of this disaccharide are shown in Table 4.

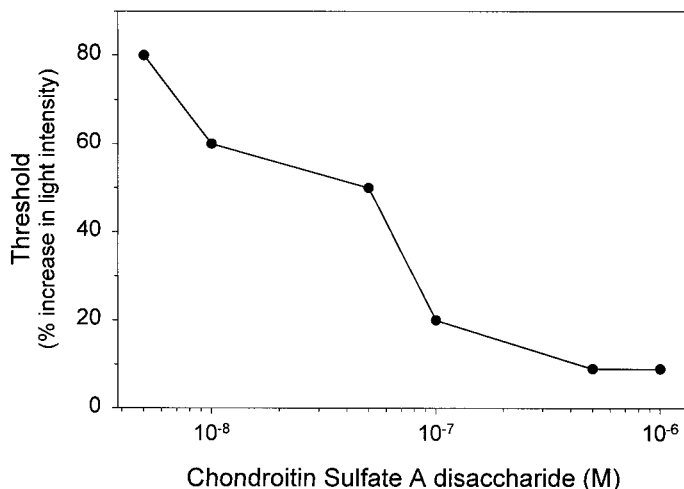


Fig. 5. The minimum percent increase in light intensity that evoked a decent response that was significantly different from the control (threshold) upon exposure to different molar concentrations of the chondroitin sulfate A disaccharide ( $\alpha$ - $\Delta$ UA-[1 $\rightarrow$ 3]-GalNAc-4S). The sample size was five for determining each threshold.

## Discussion

Although euryhaline adult brine shrimp are found in salinities from 45 psu (Kristensen and Hulscher-Emeis 1972; Persoone and Sorgeloos 1980) to 340 psu (Post and Youssef 1977), Persoone and Sorgeloos (1980) suggest that their absence from lower salinity waters is due to their inability to withstand predation. Primary predators on brine shrimp include fish (e.g., Kristensen and Hulscher-Emeis 1972; Persoone and Sorgeloos 1980), waterfowl (Lenz 1980), insects (Ramamoorthi and Tangaraj 1980; Bhargava et al. 1987) and cyclopoid copepods (Kristensen and Hulscher-Emeis 1972). Euryhaline fishes prey heavily on brine shrimp in areas with salinity discontinuities where their distributions overlap (e.g., Scelzo and Voglar 1980), and the apparent safe refuge of brine shrimp in high salinity areas can be lost after heavy rains when fish enter these areas (Kristensen and Hulscher-Emeis 1972). Kristensen and Hulscher-Emeis (1972) report-

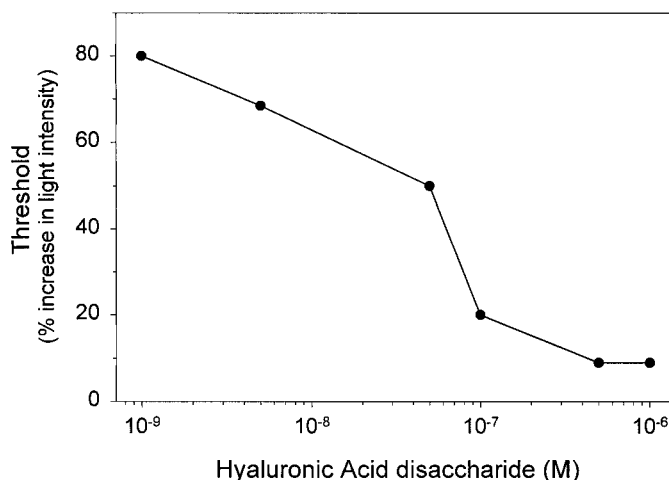


Fig. 6. The minimum percent increase in light intensity that evoked a descent response that was significantly different from the control (threshold) upon exposure to different molar concentrations of the hyaluronic acid disaccharide ( $\alpha$ - $\Delta$ UA-[1 $\rightarrow$ 3]-GlcNAc). The sample size was five for determining each threshold.

ed that optimum hatching of cysts and larval development of brine shrimp from the Antilles occurs between salinities of 5 and 60 psu with no hatching at salinities above 85 psu. Our unpublished study of hatching of *A. franciscana* found that hatching occurred at salinities between 10 and 70 psu with the highest percent hatching at 50 psu. Because euryhaline fishes occur at these lower salinities, larvae could be very susceptible to predation.

Field studies indicate that adult brine shrimp undergo nocturnal DVM (Kristensen and Hulscher-Emeis 1972; Lens 1980), in which they descend at sunrise probably to depths where light levels would reduce predation by visual predators, such as fish. Recent studies indicate that invertebrate zooplankton have a phenotypic response in which DVM is activated by kairomones from fish predators and absent or reduced in the absence of these kairomones (e.g., Dodson 1990; Neill 1990, 1992; Ringelberg 1991*a,b*; Loose 1993; Loose et al. 1993). Thus, the test hypothesis for the present study was that photoresponses of brine shrimp larvae that are involved in DVM are activated by kairomones from fish that consist of degradation products of polysaccharides that make up their external mucus. Because brine shrimp descend at sunrise in response to the relative rate of light intensity increase, activation of the descent photoresponse was used to assess the presence of fish kairomones.

Two assumptions underlying the study were that brine shrimp larvae have a similar pattern of DVM as the adults and that they are preyed upon by fish. Vertical migration of larvae is unstudied but their photoresponses are appropriate for nocturnal DVM (Forward and Hettler 1992). Small fish readily prey upon brine shrimp larvae in the laboratory (Forward and Hettler 1992). It is possible that fish kairomones activate DVM in all developmental stages of brine shrimp, but the main functional advantage is for larger individuals. Also, birds are important visual predators on many brine shrimp populations (R. Meyer pers. comm.). Because these birds may also prey on fishes, an additional source of fish kairomones may be bird fecal material.

In the present study, size-fractionated components of water, in which fish had been incubated, induced larval photoresponses involved in DVM (Table 1). Because incubation water from four different fish species induces similar photoresponses (Forward and Rittschof 1993; McKelvey and Forward 1995), and none of the test species co-occur with brine shrimp, the kairomones are common to fishes, which agrees with studies of the effects of freshwater fish kairomones on *Daphnia* (Larsson and Dodson 1993; Von Elert and Loose 1996). The active molecules are <10 kDa in size (Table 1), which also agrees with past studies with brine shrimp (Forward and Rittschof 1993; McKelvey 1997) and fish kairomones that affect DVM of *Daphnia* (Loose et al. 1993).

The test hypothesis was that active molecules originate from mucus that is sloughed off by fish. This hypothesis was supported, because fish mucus activated photoresponses involved in DVM. The minimal light intensity increase that induced a significant descent response (threshold) decreased as the fish mucus concentration increased (Fig. 3). The lowest effective concentration ( $10^{-5}$  g wet weight  $L^{-1}$ ; Fig. 3) had a sulfated sugar level of about  $0.1 \mu\text{g } L^{-1}$ . An important

consideration is the relationship of this threshold concentration to levels in natural waters. Amino-sugars are at concentrations below  $60 \mu\text{g } L^{-1}$  in seawater (Anitia and Lee 1964). Parrish and Kroen (1988) quantified the rate at which mucus was sloughed from a marine planktivorous fish (Atlantic silversides, *Menidia menidia*). Mucus was measured as sulfated sugar using the same technique as in the present study. Each fish released  $567 \mu\text{g}$  of sulfated sugar per hour when swimming at a speed of  $50 \text{ cm } s^{-1}$ . Based on their measurements, the threshold concentration (sulfated sugar =  $0.1 \mu\text{g } L^{-1} = 10^{-4}$  ppm) of mucus that activated brine shrimp photoresponses would be released by a school of 66 fish swimming at  $50 \text{ cm } s^{-1}$ .

Fish mucus is mainly composed of polysaccharide chains of repeating disaccharide units (Brimacombe and Webber 1964; Fransson 1985; Shephard 1994). The three polysaccharides studied were heparin, chondroitin sulfate, and hyaluronic acid. Disaccharides of these polysaccharides differ in their linkage (1-3 or 1-4) and the location of sulfated esters and acetyl groups (Fig. 1).

Three experimental results suggest that these types of disaccharides are present in water after fish incubation and that they activate brine shrimp photoresponses involved in DVM. First, the active molecules are <10 kDa in molecular weight (Table 1) that includes the size range for the disaccharide breakdown products. Second, incubation of the >10-kDa, <30-kDa size fraction with either heparinase lyase, chondroitinase ABC lyase (Table 2), or hyaluronidase (McKelvey 1997), which generate disaccharides, produced active molecules. Third, the >30-kDa, <100-kDa size fraction from fish water contains heparinase-like and chondroitinase-like enzymatic activities that cleave heparin and chondroitin sulfate A to produce active molecules (Table 3). The source of the enzymes could be the bacteria associated with fish mucus. This suggestion is consistent with the study of Ringelberg and Van Gool (1998), which found that production of fish kairomones was reduced in the presence of an antibiotic. The proposed type of active molecule is also consistent with the findings of Von Elert and Loose (1996) that the kairomones from a freshwater fish were hydroxylated anions but is not consistent with other aspects of their chemical identification. Kairomones in freshwater and salt water may be different due to differences in organisms and water chemistry.

Disaccharides of heparin, chondroitin sulfate A, and hyaluronic acid differed in their activity. The lowest concentrations to induce significant photoresponses were  $10^{-9}$  M for hyaluronic acid,  $5 \times 10^{-9}$  M for chondroitin sulfate A, and  $10^{-7}$  M for heparin. Both hyaluronic acid and chondroitin sulfate occur in fish mucus (e.g., van de Winkel et al. 1986), while the presence of heparin has not been reported. If heparin does not occur in fish mucus, its activity probably results from the structural similarity of the disaccharides.

Tests with compounds structurally similar to subunits of heparin, chondroitin sulfate, and hyaluronic acid suggest which functional groups are necessary for activity. Heparin disaccharide was moderately active, as it induced photoresponses at concentrations of  $10^{-7}$  M and higher (Fig. 4). If all sulfated esters were removed from the disaccharide ex-

Table 6. Order of test molecules from least to most active at inducing photoresponses involved in DVM. An increase in activity was indicated by a reduction in the minimum percent increase in light intensity necessary to induce a descent photoresponse (threshold). NR indicates the molecule did not induce a significant response at any test intensity increase. All molecules were tested at a concentration of  $5 \times 10^{-7}$  M and each is described as either a mono- or disaccharide.

Saccharide	Description	Threshold (%)
D-Glucose	Monosaccharide	NR
D-Galactose	Monosaccharide	NR
D-Galactosamine	Monosaccharide	NR
D-Galactosamine-6-sulfate	Monosaccharide	NR
D-Glucosamine-6-sulfate	Monosaccharide	NR
$\alpha$ - $\Delta$ UA-[1 $\rightarrow$ 4]-GlcN	Disaccharide	NR
$\alpha$ - $\Delta$ UA-2S-[1 $\rightarrow$ 4]-GlcN-6S	Disaccharide	NR
D-Glucosamine	Monosaccharide	60
<i>N</i> -acetyl-D-galactosamine	Monosaccharide	60
<i>N</i> -acetyl-D-glucosamine	Monosaccharide	60
<i>N</i> -acetyl-D-galactosamine-4-sulfate	Monosaccharide	60
<i>N</i> -acetyl-D-galactosamine-4,6-sulfate	Monosaccharide	60
$\alpha$ - $\Delta$ UA-2S-[1 $\rightarrow$ 4]-GlcNS-6S	Disaccharide	60
D-Glucuronic acid	Monosaccharide	50
D-Glucosamine-2-sulfate	Monosaccharide	20
D-Glucosamine-2,6-sulfate	Monosaccharide	20
$\alpha$ - $\Delta$ UA-[1 $\rightarrow$ 4]-GlcNS-6S	Disaccharide	20
$\alpha$ - $\Delta$ UA-2S-[1 $\rightarrow$ 4]-GlcNS	Disaccharide	20
$\alpha$ - $\Delta$ UA-[1 $\rightarrow$ 4]-GlcNS	Disaccharide	20
$\alpha$ - $\Delta$ UA-[1 $\rightarrow$ 3]-GlcNAc	Disaccharide	9
$\alpha$ - $\Delta$ UA-[1 $\rightarrow$ 3]-GalNAc-4S	Disaccharide	9
$\alpha$ - $\Delta$ UA-[1 $\rightarrow$ 3]-GalNAc-6S	Disaccharide	9
$\alpha$ - $\Delta$ UA-2S-[1 $\rightarrow$ 3]-GalNAc-4S	Disaccharide	9
$\alpha$ - $\Delta$ UA-[1 $\rightarrow$ 3]-GalNAc	Disaccharide	9

cept the signature sulfamino group at the 2 carbon position (Fig. 1), activity was retained at concentrations down to  $5 \times 10^{-8}$  M (Fig. 4). This sulfamino group is important for activity because all disaccharides and monosaccharides with this moiety were very potent while those with any other sulfate combination but lacking the sulfamino group were inactive or much less potent (Table 4).

Tests with chondroitin mono- and disaccharides found that disaccharides were most active and that the presence of the acetylamino group (Fig. 1) was essential for activity (Table 5). All monosaccharides that lacked this acetyl group were inactive. Similarly, the acetylamino group probably contributes to the activity of derivatives of hyaluronic acid. Thus, the structure-function studies showed that disaccharides with either 1-3 or 1-4 linkages and with a modified amine were biologically active. The most potent disaccharides had an acetylated amine.

If kairomones are generated from fish mucopolysaccharides by bacterial enzymes, then kairomones should be primary polysaccharide degradation products. Polysaccharides are first degraded to disaccharides (Fransson 1985) and subsequently to monosaccharides with varying loss of functional groups. Ordering the potency of pure compounds based upon thresholds for the descent photoresponses shows that disaccharides with acetylaminines are most potent (Table 6).

Intermediate potency was observed for mono- and disaccharides with a sulfamino group. Sulfated esters had no detectable effect on activity. Lower potency compounds were the monosaccharides *N*-acetylgalactosamine, glucosamine, and glucuronic acid. Unmodified disaccharides and other monomers of the disaccharides with ester sulfates but without a modified amine were inactive, as were glucose and galactose. Thus, the potency decline reflects the general degradation sequence for mucopolysaccharides, which supports the hypothesis that kairomones are enzymatic degradation products of sulfated and acetylated fish mucopolysaccharides.

A number of future studies are needed to verify that degradation products from fish mucus serve as fish kairomones and to elucidate other types of compounds that also serve as kairomones. Fish mucus degradation products need to be tested on other zooplankton species. Specific polysaccharides and their breakdown products should be identified in predator odor water. The levels of these products in natural waters needs to be measured and related to DVM. Tests for activity of predator odor water that has had the saccharides removed should indicate whether other kairomones are present and the potential levels of activity due to fish mucus products and other molecules.

Based upon studies of neutral-basic peptide body odors generated by the action of trypsin-like serine proteinases, Rittschof (1993) predicted that other specific chemical signals should originate from the breakdown of structural molecules, such as polysaccharides. The present study supports the prediction by demonstrating that specific disaccharides and products from the digestion of mucopolysaccharides can serve as signal molecules. These molecules are attractive as signal molecules from fish because they are unstable in the environment and thus would primarily occur in areas where fish are present.

Other parallels between peptide and modified amine disaccharide signal molecules are that both molecules are effective in submicromolar ranges and there is a wide range in potency of active molecules depending upon the nature of functional groups. Neutral-basic peptides function as cues for a variety of important activities including larval release (Forward et al. 1987), larval settlement and metamorphosis (Tegtmeyer and Rittschof 1989; Zimmer-Faust and Tamburi 1994), prey location (Rittschof 1985), shell location by hermit crabs (Kratt and Rittschof 1991), and vasodilation and leukocyte attraction in higher vertebrates (Schiffman 1982). If disaccharides containing modified amines are the signal component for another widespread chemoresponse system, then disaccharides should function to cue behaviors and processes in a similarly wide range of organisms. Thus, we predict that other fish prey will respond behaviorally to these cues.

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