

Photobehavior as an inducible defense in the marine copepod *Calanopia americana*

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Abstract

The photoresponses of *Calanopia americana* involved in diel vertical migration (DVM) were tested after exposure of copepods to the following predator kairomones: (1) crude body mucus from fish (*Fundulus heteroclitus*), (2) crude body mucus from ctenophores (*Mnemiopsis leidyi*), and (3) <10-kDa *F. heteroclitus* odor. Crude mucus (10% v/v) from fish and ctenophores and <10-kDa fish odor at several concentrations (0.01–10% v/v) either shifted or removed the ascent response of *C. americana* to relative rates of irradiance decrease that occur at sunset. The estimated concentrations of uronic acids and sulfated glycosaminoglycans in the test solutions are consistent with bioactive kairomone molecules being modified amino sugars. The phenotypic plasticity of photoresponses underlying DVM in this marine copepod species indicates that vertical migration in some marine crustacean zooplankton is likely an inducible defense, as reported for crustacean zooplankton from other habitats.

An “inducible defense” is defined as a phenotypic change in an organism that is caused directly by a cue associated with a biotic agent, and this defense usually decreases the likelihood of future encounters, or the effects of future attacks, by this agent (Harvell and Tollrian 1999). This defensive strategy can be interpreted as a special case of phenotypic plasticity because it involves a flexible (i.e., plastic) change in the morphological, physiological, biochemical, or behavioral phenotype. Plants, invertebrates, and vertebrates commonly use inducible defenses in response to herbivores, predators, competitors, and parasites (reviewed by Havel 1987; Harvell and Tollrian 1999).

If organisms are faced with the risk of mortality due to predation, their defensive response can be classified according to three general types of strategies: (1) an inducible defense, (2) a fixed defense, and (3) no defense at all. Arguing in terms of relative costs and benefits of behavior, Sih (1987) stated that in environments with variable predation, flexible (i.e., inducible) predator avoidance behaviors would be optimal, whereas fixed avoidance behaviors are best suited for stable predation environments. Harvell and Tollrian (1999) cited the following criteria for an inducible defense: (1) variable predation risk, (2) the defense is useful in avoiding/escaping the predator, (3) there is a cost associated with the defense, and (4) there is a reliable cue signaling the presence of danger.

Diel vertical migration (DVM) of zooplankton is an example of a predator avoidance strategy that is highly variable between locations and in time (Ohman 1990; Frost and Bollens 1992; Dagg et al. 1997). While DVM was previously

thought to be a genotypically fixed behavior (e.g., Sih 1987), current research demonstrating that photobehaviors underlying DVM are modified by chemical cues from predators indicates that it is better classified as a phenotypically plastic inducible defense (Ringelberg 1991; Forward and Hettler 1992; Ringelberg 1999). This seems reasonable, as the factors driving DVM meet the criteria described above for inducible defenses: (1) predation on zooplankton is variable in time and space (Burrell and Van Engel 1976; Horwood and Cushing 1978); (2) DVM is an effective predator avoidance strategy (Neill 1990); (3) there are metabolic and demographic costs associated with DVM (Frost 1988; Ohman 1990); and (4) cues signaling predation risk have been indicated to be modified amino sugars derived from predator mucus (Forward and Rittschof 1999; Rittschof and Cohen 2004).

The theoretical model for phenotypic plasticity in zooplankton DVM involves the production of chemical cues by predators, which are subsequently detected by zooplankton prey and result in altered zooplankton behavior. These chemical cues are termed kairomones, as they are understood to be interspecific signal chemicals that only benefit the receiving organism (zooplankton) in the context of the signal transmission, although potentially providing some benefit to the transmitting organism (predator) in another context (Brown et al. 1970; Dicke and Sabelis 1988). It appears that zooplankters do not respond directly to the kairomone molecules produced by a predator by orienting away from them, but rather these chemical cues modify other behaviors (e.g., DVM), which have their own proximate regulatory mechanisms (e.g., light). Phenotypically plastic DVM behavior would then result from predator kairomones affecting zooplankton behavioral responses to light, thereby altering their vertical migration pattern. This model appears to apply to DVM in both freshwater and saltwater habitats (Ringelberg 1999).

Among saltwater crustacean zooplankton, only the photoresponses for larvae of the estuarine crab *Rhithropanopeus harrisi* and the hypersaline branchiopod *Artemia franciscana* have been assayed thus far for alteration in photobehavior by predator kairomones; the effect of predator kai-

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romones on DVM-related photobehavior of a marine organism has yet to be reported. Here we examined the effect of predator kairomones on DVM-related photobehavior in the marine copepod *Calanopia americana*. This copepod species inhabits subtropical to tropical coastal waters from Cape Hatteras, North Carolina, east to Bermuda, and south to Brazil (Bowman 1971). In shallow water (~4 m) near the mouth of a North Carolina estuary in summer, *C. americana* undergoes twilight DVM: it ascends to the surface at sunset, descends to near the bottom around midnight, makes a second ascent to the surface in the latter half of the night, and then descends to near bottom at sunrise (Cohen and Forward in press a). A redundant set of proximate cues underlies DVM in *C. americana*, including behavioral responses to exogenous light cues and an endogenous rhythm. For example, the ascent of *C. americana* at sunset results from (1) upward swimming at low absolute irradiance levels, (2) upward swimming during fast relative rates of irradiance decrease, and (3) the active phase of an endogenous vertical migration rhythm (Cohen and Forward in press b).

In the present study, the behavioral response of *C. americana* to relative rates of irradiance decrease was used as a bioassay for kairomone activity. Kairomones included body odor of the fish *Fundulus heteroclitus* as well as mucus of both *F. heteroclitus* and the ctenophore *Mnemiopsis leidyi*. In summer and fall, these species are abundant in estuarine/coastal areas of the southeastern United States inhabited by *C. americana* (Hettler 1989; Purcell et al. 2001), and both prey upon copepods (Kneib and Stiven 1978; Kremer 1979; Purcell and Decker 2005). Furthermore, kairomones from these species are known to alter DVM-related photoresponses in estuarine and hypersaline zooplankton species (reviewed by Rittschof and Cohen 2004). It was hypothesized that (1) predator kairomones remove or shift the ascent response of *C. americana* to relative rates of irradiance decrease occurring at sunset; and (2) predator kairomones active in altering *C. americana* DVM-related photobehavior contain biochemical components common to mucus, such as modified amino sugars (uronic acids and sulfated glycosaminoglycans [GAGs]) at nmol L^{-1} to $\mu\text{mol L}^{-1}$ concentrations.

Material and methods

Copepod collection—*Calanopia americana* were captured using a stationary 333- μm mesh 0.75-m plankton net set prior to maximum current on night-time flood tides near Beaufort Inlet, North Carolina (34°4'N, 76°4'W). All net samples were immediately brought to the laboratory, where adult female *C. americana* were sorted under a dissecting microscope. Adult female copepods were used exclusively to reduce any variations due to sex and growth stage (age). Female *C. americana* are larger than males as adults, and female copepods in general have greater energy requirements due to egg production and are more likely to use DVM as a predator avoidance strategy (De Robertis et al. 2000).

Sorted copepods were placed in aged 100-kDa filtered offshore seawater (salinity 36). Aged 100-kDa filtered seawater

(FSW) was prepared by septic filtration (A/G Technology, model UFP-100-C-4X2A) of seawater collected ~24 km offshore of Beaufort Inlet (North Carolina) to remove biologically active molecules larger than 100 kDa, with subsequent aging for at least 1 week. This process produces seawater with a consistent chemical composition that does not alter crustacean photoresponses (Forward and Rittschof 2000). Copepods were fed 1.5 ml algal solution (3 ml concentrated Brine Shrimp Direct brand Tahitian Blend algal paste in 22 ml FSW) and allowed to acclimate overnight to a temperature of 23°C under gentle aeration. The ambient L:D cycle was maintained.

Preparation of test solutions—Mucus experiments: Ctenophores (*Mnemiopsis leidyi*) (five individuals, 5–6 cm in length, average weight = 16.3 g) were collected from the Newport River estuary (North Carolina) using a dip net. Killifish (*Fundulus heteroclitus*) (five females, 8–11 cm in length, average weight = 18.4 g) were collected in minnow traps from a tidal creek of the Newport River estuary. Only animals without observable morphological and behavioral abnormalities were used. These species were selected because both are found in the Newport River estuary, both are known to prey on copepods in estuaries (Kneib and Stiven 1978; Kremer 1979; Purcell and Decker 2005), and both generate kairomones that alter photobehaviors of estuarine and hypersaline crustacean zooplankton (Forward and Rittschof 1999 2000; Cohen and Forward 2003). Mucus was collected from *M. leidyi* and *F. heteroclitus* using the methods described in Cohen and Forward (2003) and Forward and Rittschof (1999). Briefly, each ctenophore was rinsed in FSW, placed on a preweighed Kimwipe tissue, rolled from side to side two times, and then removed. Ctenophore external mucus remained on the tissues with little observable body tissue contamination. For fish, the dorsal surface of each was wiped two times with a preweighed Kimwipe. For both ctenophores and fish, mucus-laden tissues were weighed, added to 100 ml FSW, and shaken on an orbital shaker at 1,000 rpm for 40 min (26°C). The solution was decanted, the tissues squeezed with a latex-gloved hand, and the final solution frozen until use. The crude mucus solutions were assayed for total protein (BCA; Pierce Biotechnology), total sugar (Dubois et al. 1956), uronic acids (Yoon et al. 2002), and sulfated GAGs (Farndale et al. 1986).

Three hours prior to sunset on the day following collection, groups of 50 copepods were added to an acrylic cuvette (4 × 4 × 5 cm) filled with one of three test solutions: (1) FSW, (2) 10% (v/v) *F. heteroclitus* mucus (1.7 g wet weight mucus L⁻¹), or (3) 10% (v/v) *M. leidyi* mucus (13.5 g wet weight mucus L⁻¹). As the Kimwipe procedure itself does not affect crustacean photobehavior (Forward and Rittschof 1999), FSW served as a control solution free of predator odor. Mucus solutions were prepared from thawed crude mucus diluted with FSW on the day of each trial. A 10% concentration was selected because it was greater than or equal to fish and ctenophore mucus concentrations known to alter photobehavior in other crustacean zooplankton species (Forward and Rittschof 1999, 2000; Cohen and Forward 2003). Copepods were kept for at least 1 h under cool-white fluorescent lighting (~5 × 10¹⁹ photons m⁻² s⁻¹), after which

they were dark-adapted for 2 h in a light-tight box prior to testing their photoresponses (see below).

<10-kDa Fish odor experiments—Twelve *F. heteroclitus* (7–9.5 cm in length, average weight = 15.2 g), were collected as described previously. Fish were incubated for 5 min at 23°C in 500 ml FSW. The time-corrected concentration was 1,824 g min L⁻¹, which is similar to concentrations known to alter photobehavior in crustacean zooplankton (Forward and Rittschof 1999; Cohen and Forward 2003). The incubation water was decanted and vacuum filtered (Whatman GF/F) to remove any particulate material (e.g., feces, scales, etc.). The GF/F filtrate was immediately subjected to size fractionation in a stirred ultrafiltration cell (Amicon model 8400) under pressurized nitrogen gas (2.75 × 10⁵ Pa). Ultrafiltration was conducted at 4°C to minimize degradation of fish odor by bacterial enzymes. A series of ultrafiltration membranes (Amicon Diaflo) having nominal molecular weight limits of 100 kDa (YM100), 30 kDa (YM30), and 10 kDa (YM10) were used sequentially according to the instructions of the manufacturer. GF/F-filtered fish odor (500 ml) was initially passed through a 100-kDa membrane. The <100-kDa filtrate was passed through a 30-kDa membrane. The <30-kDa filtrate was passed through a 10-kDa membrane. The <10-kDa fraction (<10-kDa fish odor) was frozen at -80°C until use. This size fraction was used in experiments because active kairomone molecules in fish odor were shown to be <10 kDa using an *A. franciscana* bioassay (McKelvey and Forward 1995; Forward and Rittschof 1999). The <10-kDa fish odor fraction was assayed for total protein, total sugar, uronic acids, and sulfated GAGs, as described previously.

Three hours prior to sunset on the day following collection, groups of 50 copepods were added to beakers (50 ml) filled with 30 ml of <10-kDa fish odor at one of five concentrations: 0, 0.01, 0.1, 1, and 10% (v/v). Solutions were prepared from thawed <10-kDa fish odor diluted with FSW on the day of each trial. Copepods were kept for 2 h under cool-white fluorescent lighting (~5 × 10¹⁹ photons m⁻² s⁻¹), after which they were dark-adapted for at least 1 h in a light-tight box. The period of dark adaptation was less than for experiments with mucus (2 h), but crustacean visual systems should be sufficiently dark-adapted for maximal photoresponsiveness after 1 h (Autrum 1981). Each group of copepods was transferred to an acrylic cuvette (4 × 4 × 5 cm) under dim red light prior to testing their photoresponses.

Photoresponse testing procedures—Copepod photoresponses were tested after the time of sunset in the field, when copepods would normally be ascending in the water column (Cohen and Forward in press *a*). A cuvette of dark-adapted *C. americana* was placed at the horizontal center of a water bath (50 × 50 × 25 cm) with inside walls painted flat black. The bath was filled with deionized water at a level just below the upper edge of the cuvette. Light entered the bath from the top and passed through a white acrylic diffuser plate, creating a uniform overhead light field with the walls of the bath outside the critical angle (zenith ± 48.6°), as viewed from the bottom of the cuvette. Testing photobehavior in this type of apparatus is important because the light

field simulates the normal underwater angular light distribution: a relatively bright light overhead with diffuse surrounding irradiance beginning about 50° from the vertical and a darker bottom. Zooplankton display abnormal photobehaviors in highly directional light fields; therefore, if behavior in the laboratory is to be relevant to behavior in the field, an apparatus like the one described is required (Forward 1988). The light source was a 750-W incandescent bulb, with irradiance controlled using both fixed neutral-density filters and a circular variable neutral-density filter wheel (Oriol Instruments), and spectral composition was limited to blue-green wavelengths by a Corning No. 4-96 filter (Kopp Glass). The spectral sensitivity of *C. americana* encompasses those wavelengths maximally transmitted by the blue-green filter (Cohen and Forward 2002). Irradiance calibration of the laboratory apparatus was in units of photons m⁻² s⁻¹ (at 500 nm; EG&G model 550 radiometer).

Copepods were light-adapted at 8 × 10¹³ photons m⁻² s⁻¹. This adaptation level was used because it was greater than the visual threshold of dark-adapted *C. americana* (1.3 × 10¹² photons m⁻² s⁻¹; Cohen 2004), but not so high as to evoke a cessation of swimming associated with irradiances greater than ~1 × 10¹⁵ photons m⁻² s⁻¹, which results in copepods remaining on the bottom of the cuvette (Cohen and Forward in press *b*). Irradiances of ~8 × 10¹³ photons m⁻² s⁻¹ are present at the bottom of the Newport River estuary (collection site) within 30 min of sunset, when the initial ascent of copepods in the water column occurs (Cohen and Forward in press *a*).

After 15 min of light adaptation, relative rates of irradiance-decreased stimuli were presented. Stimuli were continuous rates of decrease lasting 20 s created by rotating the circular variable neutral-density filter wheel with a variable speed motor. The wheel was calibrated for relative rates of irradiance change (RRC) by the following equation (Rinzelberg 1964):

$$\text{RRC} = \frac{\text{Ln}\left(\frac{I_f}{I_i}\right)}{dt}$$

where, I_f and I_i are the final and initial irradiances, respectively, and dt is the change in time in seconds. The unit for relative rates of irradiance change is s⁻¹. For initial treatments with FSW and 10% crude mucus solutions, stimuli were presented in the following order: -0.0021, -0.0032, -0.0046, -0.0058, and -0.0068 s⁻¹. After each stimulus, the wheel was returned to its prestimulus position, and copepods were given 5 min at the adaptation irradiance prior to the next stimulus. Preliminary experiments determined that this interval between stimuli did not alter the response upon repeated stimulation at intermediate rates of decrease. Subsequent treatments with <10-kDa fish odor exposed copepods to a single stimulus (-0.0046 s⁻¹) that is near the relative rate of irradiance decrease at the time during sunset when copepods would be making their initial ascent in the water column (~-0.0042 s⁻¹; Cohen and Forward in press *a*). Four replicate trials were conducted after the time of sunset in the field for each crude mucus and <10-kDa fish odor treatment.

Movement of copepods during the experiments was recorded using a closed-circuit video system, with illumination by far-red light (maximum transmission = 774 nm). Far-red light does not alter or induce photoresponses in *C. americana* (Cohen and Forward 2002). Aspects of swimming behavior and orientation were later analyzed from video recordings using a PC-based motion analysis system (CellTrak software, Motion Analysis). Swimming behavior was analyzed by determining the mean angular direction of movement in the XY-plane for copepods in the field of view (~25 individuals) from digitized video recordings during a 3-s interval 20 s prior to each stimulus (control) when copepods were exposed to light-adaptation conditions (8×10^{13} photons $\text{m}^{-2} \text{s}^{-1}$) and during a 3-s interval beginning 6 s after the start of a stimulus (response). Most copepods oriented in a significant direction during the analysis intervals (Rayleigh's z , $\alpha = 0.01$); those that did not were excluded from subsequent analyses because movement was random. The percentage of orienting copepods swimming upward toward the stimulus light $\pm 30^\circ$ (% ascending) was determined. An increase in the percentage of copepods ascending relative to the control values indicated a response to the light stimulus.

Experiments with mucus solutions had a repeated-measures design; groups of copepods received multiple relative rates of irradiance change, with behavior observed prior to and during each stimulus. If a one-factor repeated-measures analysis of variance (ANOVA) indicated no difference in the control percentage of copepods ascending during constant irradiance for a given treatment solution, control values for all stimuli were pooled, yielding a single control mean and standard error. Response data were then analyzed using a one-factor repeated-measures ANOVA including the control as an additional stimulus (Zar 1999). Multiple comparisons were done using a Dunnett's test versus the control stimulus ($q'_{0.05(1)}$ 34,6; Zar 1999). A one-tailed statistical test was used because relative rates of irradiance decrease (as at sunset) were expected to increase the percentage of copepods ascending relative to the control.

Experiments with <10-kDa fish odor solutions were designed as paired observations of the ascent response prior to and during a single relative rate of irradiance change stimulus (-0.0046 s^{-1}). Statistically significant ascent responses were determined by comparing the percentage of copepods ascending during the stimulus with the percentage ascending during paired prestimulus controls (paired t -test, $t_{0.01(1)}$ 3; Zar 1999). Significance was determined at the $\alpha = 0.01$ level (Bonferroni's adjustment; Zar 1999) as five independent statistical tests were conducted, one for each <10-kDa fish odor concentration. A one-tailed statistical test was again used.

Field measurement of relative rates of irradiance change—Atmospheric relative rates of irradiance change during sunset were measured near the copepod collection site on 19 September 2002. The sky was mostly clear, with scattered clouds. Measurements were made 1 m above the surface of the water. Irradiance was measured with a quantum cosine PAR sensor (LI-COR model 190SA), filtered with the same blue-green glass filter used in laboratory experiments. Relative rates of irradiance change were calculated as described previously.

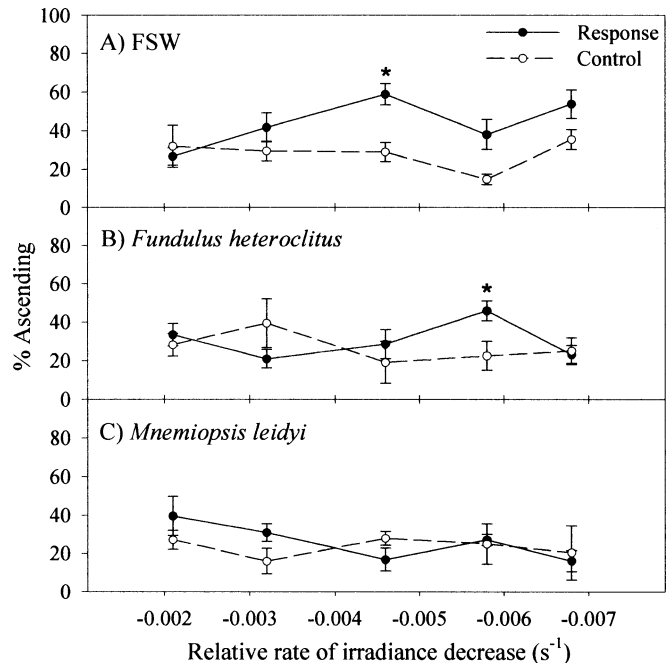


Fig. 1. *Calanopia americana* responses to relative rates of irradiance decrease with exposure to fish and ctenophore mucus. Copepods were exposed to (A) 100 kDa-filtered seawater (FSW), (B) 10% (v/v) *F. heteroclitus* mucus (1.7 g wet weight mucus L^{-1}), or (C) 10% (v/v) *M. leidyi* mucus (13.5 g wet weight mucus L^{-1}) for 3 h prior to photoresponse testing. The percentage of dark-adapted copepods swimming upward toward the stimulus light $\pm 30^\circ$ (% ascending) is plotted as a function of the relative rate of irradiance decrease. Ascent responses during the light stimuli are denoted by filled circles connected with a solid line, whereas open circles connected by a dashed line denote control responses prior to each stimulus. Means and standard errors are plotted ($n = 4$). Asterisks show responses that are significantly greater than the pooled controls (one-factor repeated measures ANOVA with a Dunnett's test for multiple comparisons, $q'_{0.05(1)}$ 34,6). Panel A is replotted from Cohen and Forward (in press *b*).

Results

Mucus treatments affected the ascent response exhibited by *C. americana* upon exposure to relative rates of irradiance decrease simulating those occurring during sunset (Fig. 1). The percentage of copepods ascending did not differ significantly among prestimulus controls for any of the treatments (FSW: $F_{4,12} = 1.750$, $p = 0.204$; *F. heteroclitus* mucus: $F_{4,12} = 0.386$, $p = 0.590$; *M. leidyi* mucus: $F_{4,12} = 0.727$, $p = 0.815$). For FSW (Fig. 1A), significant differences existed among ascent responses to rate-of-change stimuli ($F_{5,15} = 3.662$, $p = 0.023$). Only at -0.0046 s^{-1} was the ascent response significantly greater than the pooled controls ($q' = 2.738$, $\text{df} = 34$, $p < 0.05$). For *F. heteroclitus* mucus (Fig. 1B), significant differences existed among ascent responses to rate-of-change stimuli ($F_{5,15} = 2.956$, $p = 0.047$), but ascent responses significantly greater than the pooled controls only occurred at -0.0058 s^{-1} ($q' = 2.563$, $\text{df} = 34$, $p < 0.05$). For *M. leidyi* mucus (Fig. 1C), ascent responses to rate-of-change stimuli were not significantly different from

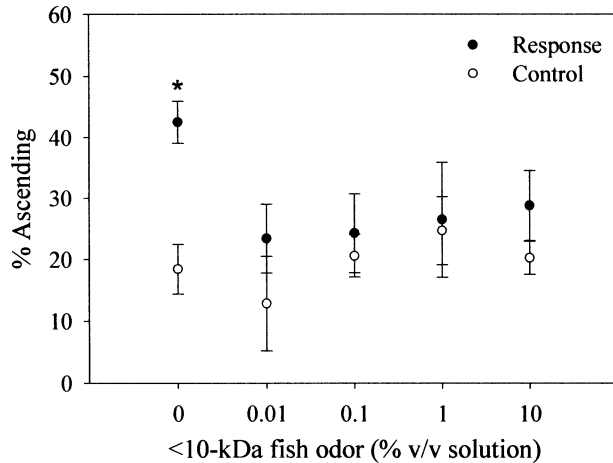


Fig. 2. *Calanopia americana* responses to a single relative rate of irradiance decrease stimulus with exposure to <10-kDa fish odor. Copepods were exposed to 0.01, 0.1, 0.1, or 10% (v/v) solutions of <10-kDa *F. heteroclitus* odor (<10-kDa fish odor) for 3 h prior to testing photoresponses upon a -0.0046 s^{-1} stimulus. Treatment 0% is FSW. The percentage of dark-adapted copepods swimming upward toward the stimulus light $\pm 30^\circ$ (% ascending) is plotted for each test solution. Ascent responses during the light stimuli are denoted by filled circles, whereas open circles denote control responses prior to each stimulus. Means and standard errors are plotted ($n = 4$). Asterisks show responses to the stimulus that are significantly greater than paired prestimulus controls (paired t -test, $\alpha = 0.01$ after Bonferroni's adjustment for multiple comparisons).

one another and from the pooled prestimulus control ($F_{5,15} = 2.425$, $p = 0.084$).

Exposure of *C. americana* to <10-kDa *F. heteroclitus* odor (<10-kDa fish odor) resulted in a loss of the ascent response (Fig. 2). Copepods exposed to FSW (0%) responded to a -0.0046 s^{-1} relative irradiance decrease stimulus, with a significantly higher percentage of ascent than was observed during the prestimulus control ($t = 6.400$, $df = 3$, $p = 0.008$) (Fig. 2). This single relative rate of irradiance decrease was used because it evoked a strong response in the previous experiment (Fig. 1A). Copepods exposed to <10-kDa fish odor solutions at concentrations of 0.01% ($t = 1.053$, $df = 3$, $p = 0.370$), 0.1% ($t = 0.420$, $df = 3$, $p = 0.703$), 1% ($t = 0.163$, $df = 3$, $p = 0.881$), and 10% ($t = 1.184$, $df = 3$, $p = 0.322$) had similar ascent responses during the stimulus and prestimulus control (Fig. 2).

Atmospheric RRC near the copepod collection site gradually became faster prior to and just after sunset, then slowed (Fig. 3). RRC changed rapidly ~ 25 to 35 min after sunset, becoming maximally negative ~ 30 min after sunset (-0.0058 s^{-1}), after which RRC slowed.

Estimated quantities of several biochemical components in 100% (v/v) solutions of crude *F. heteroclitus* mucus (fish mucus), crude *M. leidyi* mucus (ctenophore mucus), and <10-kDa *F. heteroclitus* odor (<10-kDa fish odor) are shown in Table 1. Total protein was highest in fish mucus, with estimated total protein 6.8-fold higher than ctenophore mucus, and 4.5-fold higher than <10-kDa fish odor. Similarly, estimated total sugar in fish mucus was 2.4-fold higher than ctenophore mucus and 3.1-fold higher than <10-kDa

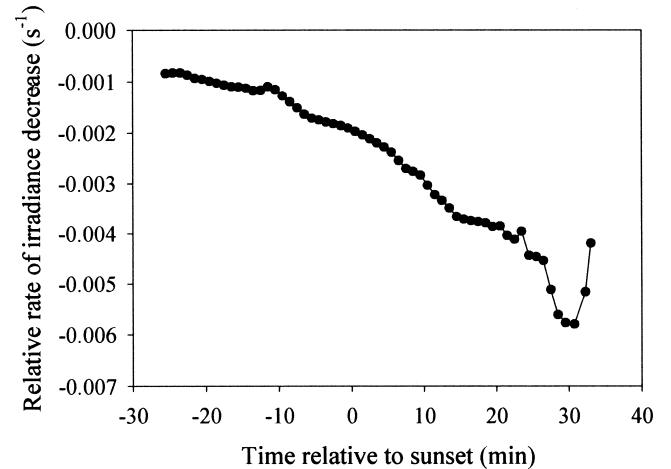


Fig. 3. Relative rate of irradiance decrease at sunset. Atmospheric measurements of relative rate of irradiance change 1 m above the surface of the Newport River estuary (Beaufort, North Carolina) during sunset on 19 September 2002. Relative rate of irradiance decrease (s^{-1}) is plotted as a function of time relative to sunset (min), where 0 min = sunset (19:08 h).

fish odor. Estimates of uronic acids were again highest in fish mucus, with the difference between fish and ctenophore mucus (7.6-fold) exceeding that between fish mucus and <10-kDa fish odor (1.7-fold). Estimates of sulfated GAGs were similar for fish and ctenophore mucus, but were below the level of detection in <10-kDa fish odor.

Molar concentrations of uronic acid and GAGs were calculated for the 10% fish and ctenophore mucus test solutions and for the 0.01–10% <10-kDa fish odor solutions from the mean values of undiluted solutions in Table 1. Calculations of uronic acid concentration used the formula weight for glucuronic acid (194.1), while calculations of GAG used the formula weight for chondroitin sulfate A disaccharides (503.3). The concentration of uronic acid in 10% mucus solutions was 2.0×10^{-6} and $2.7 \times 10^{-7} \text{ mol L}^{-1}$ for 10% fish and ctenophore mucus, respectively (Table 2). Uronic acid concentration in <10-kDa fish odor solutions ranged from 1.2×10^{-10} (0.01%) to $1.2 \times 10^{-7} \text{ mol L}^{-1}$ (10%) (Table 2). GAG concentration was $3.2 \times 10^{-6} \text{ mol L}^{-1}$ for

Table 1. Biochemical analysis of total protein, total sugar, uronic acids, and sulfated glycosaminoglycans (GAG) in kairomone solutions. All values are the means of duplicate measurements in $\mu\text{g ml}^{-1}$, with standard errors in parentheses. Undiluted (100% v/v) solutions of crude mucus and <10-kDa fish odor were assayed in order to maximize the amount of material in each sample. A value of 0 $\mu\text{g ml}^{-1}$ means that the quantity of a given analyte was below the level of detection for the assay.

Assay	<i>F. heteroclitus</i>	<i>M. leidyi</i>	<10 kDa
	mucus	mucus	<i>F. heteroclitus</i> odor
Total protein	90.76 (4.14)	13.34 (1.76)	19.95 (1.95)
Total sugar	19.13 (1.27)	8.07 (0.42)	6.20 (4.41)
Uronic acids	4.03 (1.56)	0.53 (0.53)	2.41 (0.30)
GAG	15.91 (0.04)	15.89 (1.76)	0

Table 2. Concentration of uronic acids and sulfated glycosaminoglycans in biologically active kairomone test solutions. Values are the estimated molar (mol L^{-1}) concentration of either uronic acid (based on FW 194.1 for glucuronic acid) or sulfated glycosaminoglycans (GAG) (based on FW 503.3 for chondroitin sulfate A disaccharides) calculated from the assay means in Table 1. For values of 0 mol L^{-1} , the concentration of a given analyte was below the level of detection for the assay.

Test solution	Uronic acids	GAG
10% <i>F. heteroclitus</i> mucus	2.0×10^{-6}	3.2×10^{-6}
10% <i>M. leidy</i> mucus	2.7×10^{-7}	3.2×10^{-6}
0.01% <10-kDa <i>F. heteroclitus</i> odor	1.2×10^{-10}	0
0.1% <10-kDa <i>F. heteroclitus</i> odor	1.2×10^{-9}	0
1% <10-kDa <i>F. heteroclitus</i> odor	1.2×10^{-8}	0
10% <10-kDa <i>F. heteroclitus</i> odor	1.2×10^{-7}	0

both 10% fish and ctenophore mucus and was not calculated for <10-kDa fish odor because GAG was below the level of detection for the assay (Table 2).

Discussion

The ultimate evolutionary cause of DVM of zooplankton is commonly thought to be predator avoidance (Lampert 1993; De Meester et al. 1999). Recent evidence for this hypothesis has come from studies on the proximate physiological basis of migration behavior in freshwater, estuarine, and hypersaline zooplankton. Numerous studies indicate that predator kairomones alter photobehaviors of zooplankton underlying DVM, thereby inducing DVM in previously non-migrating organisms or enhancing existing migration behavior (increased migration amplitude, delayed ascent at sunset, advanced descent at sunrise) when kairomones are present (Ringelberg 1991; Forward and Hettler 1992; Ringelberg 1999). However, evidence for the alteration of DVM-related photobehaviors in marine zooplankton has been absent. For vertically migrating marine zooplankton species like the copepod *C. americana*, which respond to relative rates of irradiance decrease at sunset with an ascent response and a subsequent rise in the water column (Cohen and Forward in press a,b), the predicted effect of kairomones on photobehavior would be (1) shifting the threshold rate of irradiance change required for induction of the ascent response to a faster rate, thereby delaying the ascent to later in sunset, when the fastest rates of change occur, and reducing exposure of zooplankters to surface-dwelling visual predators during daylight and twilight, or (2) removal of the ascent response altogether, thereby inhibiting the ascent and reducing exposure of zooplankters to all predators in the upper water column.

The present laboratory study supports these predictions. Exposure to mucus of a visual predator, the estuarine fish *F. heteroclitus*, shifted the *C. americana* ascent response from -0.0046 s^{-1} to a faster relative rate of irradiance decrease (-0.0058 s^{-1} , Fig. 1B). This faster rate of irradiance change occurred later in sunset than slower rates of change at the site where copepods were collected (Fig. 3). Concentrations of <10-kDa *F. heteroclitus* odor from 0.01–10% removed

the ascent response at -0.0046 s^{-1} (Fig. 2), but it was not determined if the ascent response was shifted to faster rates of change. Exposure of copepods to mucus of a nonvisual predator, the ctenophore *M. leidy*, removed the ascent response altogether (Fig. 1C).

The effect of predator kairomones on DVM-related photobehaviors has been reported for only three crustacean species in addition to *C. americana*, all from different habitats, with each species differing somewhat in terms of how kairomones alter photobehavior. For the freshwater cladocerans *Daphnia* spp., odor kairomones from fish (*Perca fluviatilis*) activate previously absent descent responses at sunrise and ascent responses at sunset upon relative rates of irradiance change if food levels are sufficiently high (Ringelberg 1999). For zoea of the estuarine brachyuran crab *Rhithropanopeus harrisi*, the descent response (with an isolume) at sunrise occurs at lower absolute irradiances when larvae are exposed to fish kairomones derived from *F. heteroclitus* mucus (Forward and Rittschof 2000). The effect of kairomones on the ascent response of *R. harrisi* to relative rates of irradiance decrease at sunset has not been reported. For the hypersaline anostracan *Artemia franciscana*, exposure to fish kairomones derived from *Brevoortia tyrannus* (menhaden) odor results in the initiation of a previously absent descent response upon relative rates of irradiance increase at sunrise, while the ascent response upon relative rates of irradiance decrease at sunset is not activated by predator kairomones but rather by starvation (Forward and Hettler 1992). The descent response of *A. franciscana* is similarly affected by exposure to fish odor kairomones from *F. heteroclitus* and *Lagodon rhomboides* (pinfish). Regarding invertebrate predators, *Mnemiopsis leidy* odor affects the *A. franciscana* descent response, but odor from *Callinectes sapidus* (blue crab) megalopae and *Sagitta elegans* (chaetognath) does not (McKelvey and Forward 1995). While few predator and prey species have been studied, it seems that both visual (fish) and nonvisual (ctenophore) zooplanktivorous predators produce kairomones affecting DVM-related photobehaviors in their zooplankton prey.

Kairomones from the fish *F. heteroclitus* and the ctenophore *M. leidy* were used as representative visual and nonvisual predators, respectively, in the present study because their odor and mucus alters zooplankton photobehavior and because they are common predators on copepods in the estuarine habitat from which *C. americana* was collected. *F. heteroclitus* is a dominant member of estuarine and salt-marsh nekton and is abundant in North Carolina estuaries when *C. americana* is present in summer and fall (Hettler 1989). Likewise, *M. leidy* is a common member of the estuarine and coastal plankton along the southeastern United States, frequently forming dense blooms in late summer and fall (Purcell et al. 2001). These organisms prey on copepods in very different ways. *F. heteroclitus* is a diurnally active visual predator (Clark et al. 2003), feeding at the surface in shallow water over saltmarshes and moving into deeper water at low tide (Kneib and Stiven 1978). *M. leidy* is a voracious, nonvisual predator (Burrell and Van Engel 1976; Kremer 1979; Purcell and Decker 2005) with a variable diel vertical distribution; Miller (1974) reported *M. leidy* near the surface of Pamlico Sound (North Carolina) during the

day and evenly distributed throughout the water column at night. Another nonvisual invertebrate predator of copepods, the chaetognaths *Sagitta* spp., co-occur with *C. americana* but were not considered in the present study because their odor did not affect *A. franciscana* photobehavior (McKelvey and Forward 1995) and because field data indicate that DVM in *C. americana* does not serve to avoid *Sagitta* spp., which have a tidal migration pattern (Cohen and Forward in press a).

The shift of *C. americana* ascent responses when stimulated with relative rates of irradiance decrease after exposure to fish kairomones (Figs. 1, 2) is suggestive of an alteration in migration behavior to reduce surface residence during daylight and twilight, presumably lowering mortality risk from visual predators. *C. americana* in the Newport River estuary (North Carolina) undertake twilight DVM with daytime residence on or near the bottom, an initial ascent to the surface at sunset, and a final descent to the bottom at sunrise (Cohen and Forward in press a). With fish kairomones present, the ascent response of *C. americana* to relative rates of irradiance change cues would occur later in twilight, when relative rates of irradiance change are at their fastest (Fig. 3). Copepods then enter surface waters under low ambient light, when predation pressure from visually orienting fish feeding at the surface is reduced (e.g., Clark et al. 2003). The effect of fish kairomones on copepod photobehavior may not be limited to those produced by *F. heteroclitus*; active kairomones are likely a common constituent of fish mucus produced by many species (see below; Rittschof and Cohen 2004).

An abundance of ctenophores in the water column would reduce *C. americana* DVM, as photoresponses to rates of irradiance change at sunset are inactivated with ctenophore kairomone exposure (Fig. 1). Entering the water column later in twilight during a ctenophore bloom, as described previously for fish kairomones, should not reduce predation pressure on copepods; ctenophores do not require light to feed on copepods and have a uniform night-time vertical distribution (e.g., Miller 1974). However, reducing vertical movement at twilight, when ctenophores are abundant, by not entering the water column at night would presumably lower mortality risk. Without behavioral responses to relative rates of irradiance change, *C. americana* would still migrate upward at sunset as a result of other behaviors (absolute irradiance decreases and the active phase of an endogenous rhythm; Cohen and Forward in press b), but migration amplitude may be reduced by the loss of a major proximate ascent cue. It is not known whether the other behaviors that underlie *C. americana* DVM are affected by predator kairomones. While our laboratory experiments appear to indicate predator-specific phenotypic plasticity in copepod DVM, long-term field studies relating *C. americana* DVM with fish and ctenophore abundance and vertical distribution would aid in confirming these scenarios.

Although the present data represent the first report of an effect of predator kairomones on DVM-related photobehaviors for a marine zooplankton, several field studies indicate that DVM in marine copepods is a phenotypically plastic behavior. The proportion of the populations of marine copepods *Calanus pacificus*, *Pseudocalanus newmani*, and

Acartia hudsonica undergoing nocturnal DVM has been shown to increase with increased abundance of zooplanktivorous fish (Bollens and Frost 1989a; Bollens et al. 1992; Frost and Bollens 1992). In all cases, DVM behavior was observed to be an induced response to increased predation pressure. Bollens and Frost (1991) demonstrated that DVM is rapidly induced in *A. hudsonica* exposed to fish predators in field enclosures, indicating that DVM behavior in marine copepods is indeed phenotypically plastic. In a series of related field studies with *A. hudsonica*, Bollens and Frost (1989b) and Bollens et al. (1994) suggested that mechanical or visual cues, rather than chemical cues (kairomones), were necessary to trigger the phenotypic induction of DVM (but see Ringelberg 1995). These field studies seemingly conflict with mounting evidence from laboratory studies indicating that the photobehaviors known to cause DVM behavior in crustacean zooplankton from a diversity of habitats are mediated by predator kairomones (Forward and Rittschof 1999, 2000; Ringelberg 1999). This discrepancy may be due to the sensory system of *A. hudsonica*. The genus *Acartia* is better developed for mechanoreception than for chemoreception, which is not the case for other species of marine copepod (Bollens et al. 1994). The present results with *C. americana* demonstrate that predator kairomones do indeed alter DVM-related photobehavior, indicating that kairomone-induced DVM may be applicable to other species of marine copepods.

Kairomone molecules that alter DVM-related photobehaviors of estuarine and hypersaline crustacean zooplankton are thought to be modified amino sugars derived from predator mucus (Rittschof and Cohen 2004). Modified amino sugars are a suite of molecules consisting of sulfated, aminated, and acetylated disaccharides and larger oligosaccharides that routinely occur with uronic acids in the glycosaminoglycan polysaccharide chains of large proteoglycans (e.g., heparin, chondroitin sulfate, and hyaluronic acid; Fransson 1985). Proteoglycans are present in the external mucus of fish, where they play a structural role in hydrating the mucus layer (Shephard 1994). Both odor and body mucus from fish and ctenophores alter DVM-related photobehaviors of crustacean zooplankton (Figs. 1, 2). A similar photobehavioral alteration occurs when zooplankton are exposed to purified amino sugar disaccharides containing a uronic acid residue and a hexosamine residue modified with a sulfated and/or acetylated amino group at nmol L⁻¹ to μmol L⁻¹ concentrations (Forward and Rittschof 1999, 2000; Cohen and Forward 2003). Accordingly, it was hypothesized that predator kairomones active in altering *C. americana* DVM-related photobehavior (10% fish mucus, 10% ctenophore mucus, and 0.01–10% <10-kDa fish odor) would contain uronic acids and sulfated GAGs at nmol L⁻¹ to μmol L⁻¹ concentrations.

This hypothesis was largely supported for uronic acids, as estimated uronic acid content for all test solutions except the lowest <10-kDa fish odor concentration (0.01%, 1.2 × 10⁻¹⁰ mol L⁻¹) were in the nmol L⁻¹ to μmol L⁻¹ range (Table 2). As even this lowest <10-kDa fish odor concentration was biologically active (Fig. 2), *C. americana* may be more sensitive to kairomones than the previously tested saltwater crustacean zooplankton species (*R. harrisii* and *A. francisci*).

cana). Alternatively, the fish odor preparation may contain individual compounds, or blends of several compounds, with higher biological activity than would be predicted based on assays with pure amino sugars.

The hypothesis was also supported for sulfated GAGs in mucus test solutions but not in <10-kDa fish odor solutions. Estimated GAG content for both fish and ctenophore 10% mucus solutions was 3.2×10^{-6} mol L⁻¹, whereas GAG was not detected in <10-kDa fish odor (Tables 1, 2). While GAG may have been absent from fish odor, small oligosaccharides do not react in the DMMB assay (Farndale et al. 1986), and any GAG in the <10-kDa fish odor size fraction is likely present as small oligosaccharides. As uronic acids were present in fish odor (Tables 1, 2), and since there was biological activity in all concentrations of <10-kDa fish odor (Fig. 2), it is likely that sulfated GAGs were present in <10-kDa fish odor, but the sugar molecules were too small to be detected in the DMMB assay.

The ascent response exhibited by *C. americana* upon relative rates of irradiance decrease, which is a proximate cue underlying its DVM, is altered by predator kairomones. This phenotypic plasticity in DVM-related photobehavior indicates that DVM in *C. americana* is an inducible defense. Migration in this copepod species will likely occur with or without kairomones as a result of a redundancy in behaviors underlying DVM (i.e., responses to absolute irradiance cues and a night-active endogenous behavioral rhythm also underlie *C. americana* DVM; Cohen and Forward in press b), but predator kairomones could alter DVM by delaying the ascent to later in sunset (fish kairomones) or by reducing migration amplitude (ctenophore kairomones). In this way, the effect of kairomones on DVM behavior may in many cases be more subtle than simply inducing migration behavior in previously nonmigrating organisms. While it is not possible at this time to fully assess the generality of migration behavior as an inducible defense in the marine environment, the present study indicates that kairomone-mediated phenotypic plasticity of DVM, as proposed for crustacean zooplankton in freshwater, estuarine, and hypersaline habitats, is applicable to marine crustacean zooplankton.

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