

Orientation of larval and juvenile horseshoe crabs *Limulus polyphemus* to visual cues: Effects of chemical odors

Julie M. MEDINA, Richard A. TANKERSLEY*

Department of Biological Sciences, Florida Institute of Technology, Melbourne, FL 32901, USA

Abstract Adult horseshoe crabs *Limulus polyphemus* have long served as models for the study of vision in marine arthropods. Yet, little is known about the ability of early life history stages to detect and respond to visual cues. We examined the visually directed movements of larvae and first stage juveniles to horizons containing dark visual targets of different sizes. The study tested the hypotheses that (1) larval and juvenile crabs can detect and respond to visual targets and (2) the direction of orientation varies with the presence of chemical cues associated with settlement habitats. Orientation of larval and juvenile crabs to rectangles subtending angles from 30–330° was tested in a circular arena containing water that either lacked estuarine chemical cues (offshore water) or contained odors from aquatic vegetation or known predators. In the absence of chemical odors, larvae oriented toward and juveniles moved away from dark horizons subtending angles > 60°. When placed in water containing chemical odors from potential nursery habitats, including the seagrasses *Halodule wrightii* and *Syringodium filiforme*, crabs reversed their direction of orientation relative to their responses in offshore water. Odors from two known predators, the mummichug *Fundulus grandis* and blue crab *Callinectes sapidus*, had no effect on the orientation of larvae. Yet, juveniles responded to both odors by moving toward the visual target. Results support the hypothesis that the visual orientation of larval and juvenile horseshoe crabs changes upon exposure to habitat and predator cues and that the direction of the response undergoes an ontogenetic shift following metamorphosis [*Current Zoology* 56 (5): 618–633, 2010].

Key words Orientation, Visual cues, Chemical odors

Many mobile invertebrates inhabiting tidal and subtidal areas rely on directional orientation for survival (for reviews see Herrnkind, 1972, 1983). Orientation towards or away from environmental cues, such as visual, chemical, or tactile stimuli, help animals evade predators and avoid unfavorable environmental conditions, as well as find food, suitable habitats, and mates. Directed movements may be in response to single sources or modalities or may involve the integration of information from multiple sensory channels or inputs (Passaro and Marler, 1999). Visual cues are often employed by marine arthropods, especially crustaceans, for orientation, predator avoidance, shelter and habitat location, and prey and conspecific identification (e.g., Hazlett, 1982; Herrnkind, 1968, 1972, 1983; Forward, 1988; Diaz et al., 1995a, b, 2001; Chiussi and Diaz, 2002). However, the nature of the response (e.g., direction or intensity) to visual cues may be modified by secondary stimuli, including chemical cues from predators, prey, habitats, and conspecifics (reviewed by Ache, 1987; Atema, 1995). The integration of communication signals, such as chemical and visual stimuli, and the

simultaneous use of multisensory channels (i.e., multimodal signals) has several potential benefits, including improved detection and localization, increased information capacity compared to a single-channel signal, and enhanced protection (through redundancy) against lost signals or noisy channels (Passaro and Marler, 1999; Hazlett and McLay, 2000).

The visual system of adult horseshoe crabs *Limulus polyphemus* has long served as a model for studies of visual neuroscience, including circadian rhythms in visual systems, electrical impulses in optic nerves, and the neural code underlying visually guided behaviors (reviewed by Barlow et al., 2001; Barlow and Powers, 2003). Visual orientation plays a significant role in horseshoe crab mating. Pairs of crabs come ashore near the time of high tide, especially during spring tides, to lay and fertilize clutches of eggs in nests 10–20 cm below the sediment surface in the mid- to upper-intertidal zone (Shuster, 1982; Shuster and Botton, 1985; Brockmann, 1990; Penn and Brockmann, 1994). Male horseshoe crabs are attracted to dome-shaped objects that resemble female crabs, suggesting that vision is the

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* Corresponding author. E-mail: rtankers@fit.edu

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dominant sensory system for mate location (Barlow et al., 1982, 1984, 1987, 1988). Female crabs appear to use similar visually oriented behaviors to avoid the nesting areas of other females (Barlow et al., 1982; Powers et al., 1991). More recently, Hassler and Brockmann (2001) and Saunders et al. (2010) demonstrated that male crabs also use chemical cues, in addition to visual orientation, for mate location. Although it is unknown how crabs process or integrate visual and chemical cues during mating, the use of multiple signals is consistent with hierarchical or multimodal communication (Passaro and Marler, 1999, Saunders et al., 2010).

In contrast to the extensive literature on the visual system of adult *L. polyphemus*, relatively little attention has been given to the visual capabilities of early life history stages and its role in behavior. Like many estuarine invertebrates, the life cycle of *L. polyphemus* includes both planktonic and benthic phases. Following development in the sand, larvae (trilobites) hatch and enter the water column when nests are inundated at high tide. They remain in the plankton for 7–10 days before settling on the benthos and metamorphosing into the juvenile stage (Rudloe, 1979; Shuster, 1982; Botton and Loveland, 2003; Burton et al., 2009; Botton et al., 2010). During this period, the visual system of larval crabs undergoes rapid development. Following hatching, trilobite larvae possess one of two different types of eyes (Harzsch et al., 2006). Newly hatched larvae possess rudimentary photoreceptors capable of detecting light (Harzsch et al., 2006). Although no ommatidia are present early in the trilobite stage, the lateral eyes develop rapidly and contain up to 17 ommatidia/eye as larvae approach metamorphosis (Waterman, 1954; French, 1980; Sekiguchi et al., 1982; Meadors et al., 2001; Harzsch et al., 2006). The lateral eyes continue to grow in juveniles through the addition of new units and growth of existing ommatidia (Harzsch et al., 2006).

Following metamorphosis, juvenile horseshoe crabs generally remain in shallow, nearshore benthic habitats for up to two years before moving into deeper water (Rudloe, 1981; Brockmann and Penn, 1992; Botton et al., 2003). During this time, they frequently burrow in the substrate or reside in complex vegetative habitats, including seagrass and algae (Shuster, 1979; Ehlinger et al., 2003; Ehlinger and Tankersley, 2007, 2009; Tankersley and Boleman, unpublished data). Because *L. polyphemus* larvae undergo a shift from a planktonic to benthic existence at the time of metamorphosis, the response of trilobite larvae and post-metamorphic juvenile crabs to habitat associated cues are hypothesized to be

different. Such cues include visual stimuli and chemical odors that might signal the presence of predators or nursery habitats (e.g., seagrass and macroalgal beds).

The objective of this study was to examine the visual orientation of newly hatched *Limulus* trilobite larvae and first stage juveniles in response to horizontal targets of different sizes and to determine if their responses are influenced by the presence of chemical cues. Specifically, we tested the hypotheses that the behavioral response of larval and juvenile crabs to targets (1) undergoes an ontogenetic shift in direction following metamorphosis and (2) changes upon exposure to chemical cues that signal the presence of conspecifics, vegetation, and predators.

1 Materials and Methods

1.1 Collection of adult *Limulus polyphemus*

Adult *Limulus polyphemus* were collected from the Banana River, Melbourne, Florida (28°12'34.02"N, 80°38'16.09"W). The Banana River is one of three shallow sub-basins comprising the Indian River Lagoon. Crabs were maintained in a 750 L recirculating fiberglass holding tank with seawater (35 PSU, 22–24°C). Seawater used for maintaining adults and rearing larvae and juveniles was collected near the Florida Institute of Technology Vero Beach Marine Laboratory, Vero Beach, FL (27°40'31.83"N, 80°21'50.69"W). Since this water is pumped from a shallow coastal area about 50 meters from the beach, it was assumed to contain chemical cues and odors associated with coastal habitats. To distinguish it from other types of water used in experimental treatments, it is hereby referred to as “coastal water”.

1.2 Rearing of larval and juvenile horseshoe crabs

Larval and juvenile crabs were obtained using one of two methods. The first involved collecting recently fertilized eggs from nests deposited in either the field or the laboratory. For the second method, eggs were extracted from gravid females and artificially fertilized using the procedures described by Brown and Clapper (1981) and Sekiguchi (1988). After fertilization, eggs were rinsed with filtered seawater (35 PSU) and placed on moist paper towels in rectangular glass containers (23 cm × 33 cm × 5 cm). The density of embryos within the container was ~1.3 cm⁻². Paper towels lining the glass containers were moistened with seawater daily to prevent desiccation. Developing embryos were maintained in an illuminated, thermostatically controlled incubator (Precision Scientific Model #818, Winchester, VA) at 26 °C and a 14:10 light:dark cycle.

Following hatching, larvae were kept in glass culture bowls (19 cm diameter \times 5.6 cm; 50–100 individuals per bowl) containing \sim 1 L of coastal water (35 PSU; 24–25 °C). Larvae were separated by batch/cohort (i.e., eggs from the same female or nest) and hatching date. For all experiments, treatment groups included individuals from at least 5 different cohorts/batches. All larvae used in experiments were at least 5 days post-hatching and were assumed to possess lateral eyes with functional ommatidia (Harzsch et al., 2006). Following metamorphosis, juveniles were placed in 38 L glass aquaria containing 10 L of coastal water (35 PSU; 24–25 °C) and fine sand covering the bottom (2 cm deep) and were fed newly hatched *Artemia* nauplii daily (final concentration = 5 ml⁻¹).

1.3 Test Arena and Orientation to Visual Targets

Responses of larvae and juvenile crabs to visual stimuli were determined using a circular arena similar to the one described by Diaz et al. (2001). The arena consisted of a clear lucite outer ring (15 cm dia \times 4 cm height \times 0.9 cm thick) glued to a clear circular lucite plate (15 cm dia \times 0.9 cm thick) that served as the floor. The arena was filled with 400 ml of test water (see below for a complete description of water types) to a depth of 3 cm. A white, translucent, removable collar (15 cm dia \times 4 cm tall) was placed around the outside of the arena's outer ring. The arena was illuminated using a circular fluorescent light (23 cm dia, 30 watts) placed on the outside of the outer ring and collar. Thus, the white collar served to diffuse the light entering the arena and to obstruct the test animal's view of the outside environment. All experiments were conducted in a darkened room so that the only light stimulus was from the circular light around the outside of the arena. Visual targets were constructed from black construction paper and were placed between the outer wall of the arena and the white collar. All targets were the same height (4 cm) but were different widths and subtended regions (angles) between 30°–330° (at 30° increments) around the outer edge of the arena. To control for any bias in the chamber, the position of the target was randomized before each trial.

To introduce animals to the center of the arena, a 1.5 cm dia \times 5 cm acrylic tube was attached to the bottom of the arena near the center. The top of the tube was open and level with the floor of the arena. The bottom of the tube was fitted with a movable plunger that was used to raise test subjects to the surface of the arena. The arena was placed on top of a table containing a small hole for the central tube to pass through so that

the plunger could be operated from below.

At least 24 h prior to being used in experiments, larvae and juveniles were transferred from coastal water to offshore water (see below for complete description). Trials were initiated by placing a single larval or juvenile crab in the central tube and giving it one minute to acclimate to the experimental conditions. The crab was then introduced to the center of the arena by slowly raising the plunger. Once the crab entered the arena, its movements relative to the visual target were monitored from above using a B/W video camera (Panasonic WV-BP330) and monitor (Panasonic CT-1384Y). Once the crab reached the side of the chamber, it was removed and its position relative to the visual target (center = 0°) was recorded to the nearest 5°. Thus, crabs whose final positions were to the right or left of the center of the target were assigned values between 0°–180° or 180°–360°, respectively (Fisher, 1993; Mardia and Jupp, 2000). Crabs were considered “unresponsive” if they failed to reach the edge of the arena within 5 minutes. Preliminary trials conducted using all visual targets ($n=50$ per trial) and under diffuse fluorescent light indicated that the orientation responses of larvae and juveniles did not differ between daytime and nighttime trials. Therefore, all experiments were conducted during the day (10:00–19:00 h).

1.4 Effect of habitat, predator, and conspecific chemical odors on orientation

To determine if orientation to visual targets differed in the presence of chemical odors, responses of larval and juvenile crabs were monitored in two different types of test water – offshore and odor water. Offshore water (35 PSU; filtered $<$ 5 μ m) was collected 15 km seaward of Ft. Pierce Inlet, Ft. Pierce, Florida. The collection site was beyond the estuarine plume associated with the Indian River Lagoon and Ft. Pierce Inlet, Florida. Therefore, the water was considered to be relatively free of chemical cues and odors associated with the estuary. To determine the effect of chemical odors from potential estuarine nursery habitats on the visual orientation of crabs, the responses of crabs to visual targets in offshore water were repeated using seawater (35 PSU) containing chemical cues associated with the shoal grass *Halodule wrightii* (*H-w* odor water). Field studies indicated that *H. wrightii* serves as important settlement and nursery habitat for horseshoe crab larvae and juveniles in the Indian River Lagoon (Tankersley and Boleman, unpublished data). *H-w* odor water was produced by incubating freshly collected *H. wrightii* in offshore water for 24 hours at a concentration of 31.25 g L⁻¹. Prior

to being used in experiments, the shoal grass was removed and the water was filtered ($< 5 \mu\text{m}$). The water was then used in place of offshore water in the test arena. The orientation of larvae and juvenile crabs to visual targets subtending angles between 0° (no target)– 330° at 30° increments were recorded in offshore water and *H-w* odor water. Fifty larvae and juveniles were tested individually at each target size and water combination.

Because *H. wrightii* odor was found to influence the orientation behavior of larvae and juveniles (see Results), a second series of trials was conducted to determine if the observed behavioral response (i.e., 180° phase shift in orientation relative to controls; see Results) was concentration dependent. A 180° visual target was used for all trials. *H-w* odor water was produced following the procedures outlined above and dilutions were obtained by adding the appropriate amounts of offshore water. Concentrations ranging between 0.312 g L^{-1} and 31.25 g L^{-1} were tested at $\frac{1}{2}$ log unit increments. Separate groups of fifty larval and juvenile crabs were tested individually at each concentration. Responses of crabs to the 180° visual target in the absence of chemical cues (i.e., offshore water) served as the control.

To determine if the orientation of larval and juvenile horseshoe crabs is influenced by other chemical cues, orientation trials were repeated using chemical odors from one of three categories of sources: (1) vegetation associated with potential nursery habitats, (2) potential predators, and (3) conspecifics. In addition to *Halodule wrightii*, crabs were also tested in water containing odors from the manatee grass *Syringodium filliforme* and the drift algae *Acanthophora* spp. These vegetation cues were used because they occur in the collection estuary (Indian River Lagoon, FL) and are known to serve as potential habitats for newly settled and metamorphosed juvenile horseshoe crabs (Shuster, 1979; Tankersley and Boleman, unpublished data). Two types of predator odor water were created using blue crabs *Callinectes sapidus* and mummichugs *Fundulus grandis*. Both organisms are known predators of horseshoe crab larvae and juveniles (Botton and Shuster, 2003). Juvenile (sexually immature) horseshoe crabs were used to create conspecific odor water. Test waters from all odor sources were prepared by incubating live material (*Syringodium filliforme*, *Acanthophora* spp., *C. sapidus*, *F. grandis*, or juvenile *L. polyphemus*) in offshore water at a concentration of 31.25 g (wet weight) L^{-1} using the procedures outlined above for *H-w* odor water. Responses of crabs ($n=50$) to an 180° target in each type of odor water were compared to the re-

sponses of similar groups of animals tested in offshore water (control).

1.5 Data Analysis

Responses of crabs to visual targets were analyzed using circular statistics (Zar, 2010). A Rayleigh's test was used to test the null hypothesis that the distribution of the final positions of the crabs in the arena was uniform (i.e., no orientation) at $\alpha = 0.05$ (Oriana 3.0, Kovach Computing Services). Mean angle ($\bar{\alpha}$) and the vector length (r) were also calculated and were used to compare the direction and strength of orientation of crabs relative to the location of the visual target. Mean angles are only reported for distributions in which the circular distribution was found to be significantly different from uniform at $\alpha = 0.05$. Crabs that oriented toward the target were expected to have a mean angle of orientation of $\sim 0^\circ$. Those that oriented away from the target were expected to have a mean angle of $\sim 180^\circ$. Comparisons of angular dispersions among treatments were made using a modified Kruskal-Wallis test and associated nonparametric multiple comparisons for circular data (Zar, 2010). A log-likelihood ratio test (G-test for contingency tables) was used to compare the response (% responding in 5 min) of larval and juvenile crabs to similar visual targets (Zar, 2010).

2 Results

2.1 Orientation to visual targets

When placed in a circular arena containing offshore water and no visual target (white collar only; control condition), both *L. polyphemus* larvae and juveniles failed to exhibit directed orientation responses (Larvae: $r = 0.06$, $Z = 0.13$, $P = 0.88$, $n = 32$, Fig. 1A; Juveniles: $r = 0.09$, $Z = 0.37$, $P = 0.69$; $n = 46$, Fig. 2A). Thus, there were no apparent biases in the arena or the experimental setup. Responses were similar when larvae and juveniles were presented with targets subtending angles of 30° and 60° (Figs. 1B,C, 2B,C). Yet, larvae oriented significantly toward visual targets $\geq 90^\circ$ with mean direction of orientation near the center of the target (0°) ($\bar{\alpha}$ range: 358.8° – 19.4° ; Fig. 1D–L). In contrast, juvenile crabs oriented away from visual targets $\geq 90^\circ$ with mean angles of orientation near 180° ($\bar{\alpha}$ range: 176.8° – 195.3° ; Fig. 2D–L) relative to the center of the target. Although both larvae and juveniles reacted to targets of similar size ($\geq 90^\circ$), a significantly higher percentage of juveniles reacted to visual targets within 5 minutes (Table 1).

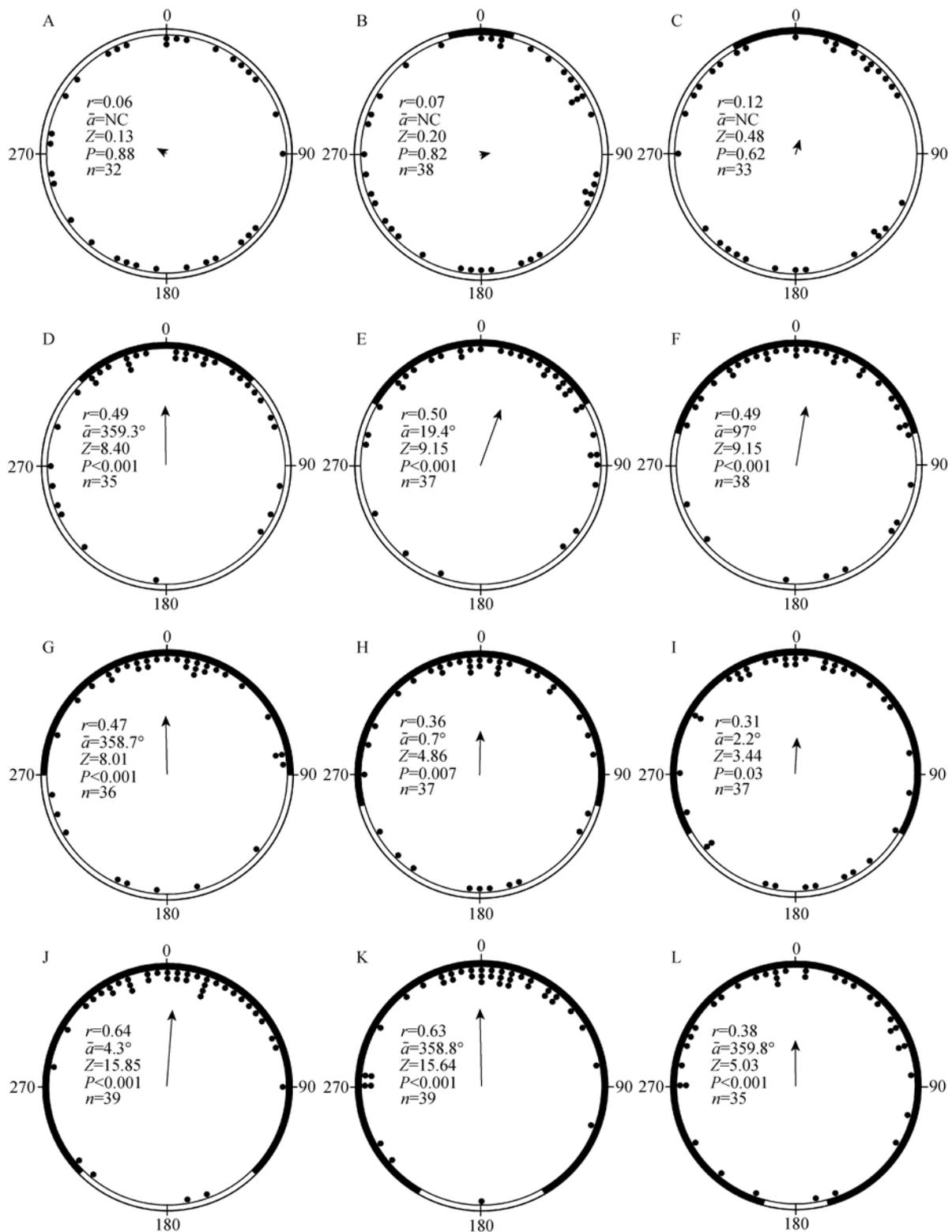


Fig. 1 Circular scatter plots of the distribution of *Limulus polyphemus* larvae in an arena in response to visual targets (black rectangles) ranging in size from 0°(no target) to 330°(A–L)

All trials were conducted in offshore water. The size and location of the target is indicated by the solid black band on the outside of the circle. Solid dots represent the directional response of individual crab larvae. The arrow in the center indicates the mean angle ($\bar{\alpha}$) of the distribution (direction). Length of the arrow is equal to the vector length (r) (radius of the circle = 1) which is a measure of the degree of dispersion and varies between 0 (dispersed) and 1 (concentrated). Rayleigh's Z statistic (Z), and associated significance (P) value, tests the null hypothesis that the circular distribution of points is uniform (i.e., no orientation). Mean angles were only calculated for distributions in which the null hypothesis was rejected at $\alpha = 0.05$. NC = not calculated; n = sample size.

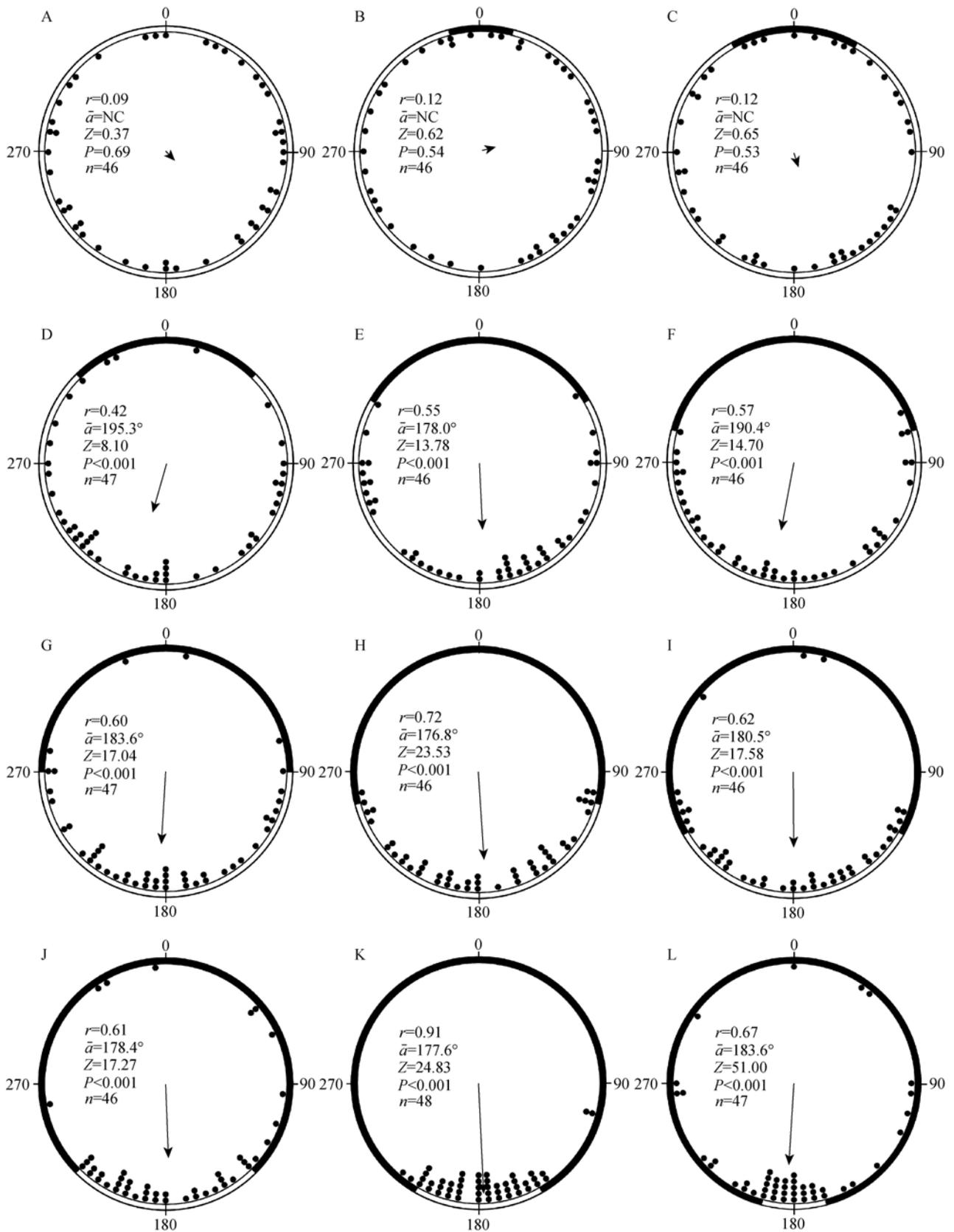


Fig. 2 Circular scatter plots of the distribution of *Limulus polyphemus* juveniles in a circular arena in response to visual targets (black rectangles) ranging in size from 0° to 330° (A–L)

All trials were conducted in offshore water. Details same as for Fig. 1.

Table 1 Comparison of the percentages of *Limulus polyphemus* larvae and juveniles (out of 50) responding to black rectangular targets subtending different angles (target size) along the outer edge of a circular arena containing offshore water or *Halodule wrightii* odor water at a concentration of 31.25 g L⁻¹

Target Size	Offshore Water % Responding				<i>Halodule wrightii</i> Odor Water % Responding			
	Larvae	Juveniles	<i>G</i>	<i>P</i>	Larvae	Juveniles	<i>G</i>	<i>P</i>
0°	64	92	12.16	<0.001	74	84	1.52	0.22
30°	76	92	4.95	0.26	76	90	3.56	0.06
60°	66	92	10.81	<0.001	74	90	3.32	0.07
90°	70	94	10.50	<0.001	78	88	1.79	0.18
120°	74	92	5.99	0.14	80	90	1.99	0.16
150°	76	92	4.95	0.26	78	88	1.79	0.18
180°	73	94	9.19	<0.001	76	92	4.95	0.26
210°	74	92	5.99	0.14	88	92	0.45	0.50
240°	74	92	5.99	0.14	80	94	4.54	0.03
270°	78	92	3.97	0.04	78	94	5.61	0.02
300°	78	96	7.79	0.01	78	94	5.61	0.02
330°	70	94	10.50	<0.001	78	88	1.79	0.18

G-tests (Log-likelihood Ratio), and associated significance (*P*) values were used to test the null hypothesis that larvae and juveniles responded equally to the same size visual target. [df (degrees of freedom) = 1 for all comparisons].

2.2 Effect of habitat, predator, and conspecific chemical odors on orientation

To test the effect of chemical cues on the visual orientation of larvae and juvenile horseshoe crabs, orientation trials were repeated using water containing chemical odor from the shoal grass *Halodule wrightii*. As in offshore water, larvae and juveniles showed no directed movements in the absence of a visual target (Figs. 3A and 4A). Larvae did not respond to 30° or 60° dark rectangles (Fig. 3B-C), but larvae were significantly oriented ($P < 0.05$) to targets subtending angles $\geq 90^\circ$ (Fig. 3D-L). Juveniles showed directed orientation to rectangles subtending angles $\geq 30^\circ$ (Fig. 4B-L). However, both larvae and juveniles were oriented in the opposite direction of their responses in offshore water (Figs. 1D-L and 2D-L). Larvae moved away from visual targets with mean directions near 180° (\bar{a} range: 168.8°–185.2°; Fig. 3D-L) relative to the center of the target. Conversely, juveniles moved toward the center of the visual targets with mean angles (\bar{a}) near 0° (range: 352.1°–9.8°; Fig. 4B-L). A significantly higher percentage of juveniles responded to visual targets for target sizes of 180°, 240°, 270° and 300° (Table 1).

The effect of chemical odors from *H. wrightii* on the orientation of both life history stages was concentration dependent. At a concentration of 9.88 g L⁻¹, larval orientation was similar to that observed at a concentration of 31.3 g L⁻¹ (i.e., away from target), yet was significantly more variable ($r = 0.80$ vs. 0.29; $Q = -47.68$, $df =$

39, 40, $P = 0.03$; Fig. 5A). At a concentration of 3.12 g L⁻¹, larval responses were no longer significantly oriented ($r = 0.03$, $Z = 0.05$, $P = 0.96$, $n = 40$; Fig. 5A). At the lowest concentrations, the direction of orientation reversed and became similar to that seen in offshore water (0.99 g L⁻¹: $\bar{a} = 7.7^\circ$, $r = 0.32$, $Z = 4.15$, $P < 0.02$, $n = 41$; 0.31 g L⁻¹: $\bar{a} = 351.7^\circ$, $r = 0.49$, $Z = 9.97$, $P < 0.001$, $n = 41$; Fig. 5A).

Juveniles also exhibited a change in the direction of orientation when exposed to decreasing concentrations of *H-w* odor water. Movements of juvenile crabs became significantly less directed (more dispersed) with decreasing concentration ($H = 20.35$, $df = 5$, $P = 0.001$; Fig. 5B), but were still significantly oriented toward the visual target ($P < 0.05$) at concentrations ≥ 3.12 g L⁻¹ (a range 356.2°–358.4°; Fig. 5B). At a concentration of 0.99 g L⁻¹, responses were no longer significantly oriented ($r = 0.14$; $Z = 4.85$, $P = 0.43$, $n = 43$; Fig. 5B). As the concentration was lowered to 0.31 g L⁻¹, the direction of orientation reversed and became similar to that seen in offshore water ($\bar{a} = 181.5^\circ$, $r = 0.47$; $Z = 10.07$, $P < 0.001$; $n = 45$; Fig. 5B).

Upon exposure to other potential nursery habitats, as well as predators and conspecifics, both larvae and juveniles exhibited orientation responses that varied with the source of the chemical odor (Table 2). Responses to a 180° target in the presence of manatee grass *S. filliforme* odor (Fig. 6A, B) were similar to those recorded in *H-w* odor water (Figs. 3G and 4G). Larvae oriented

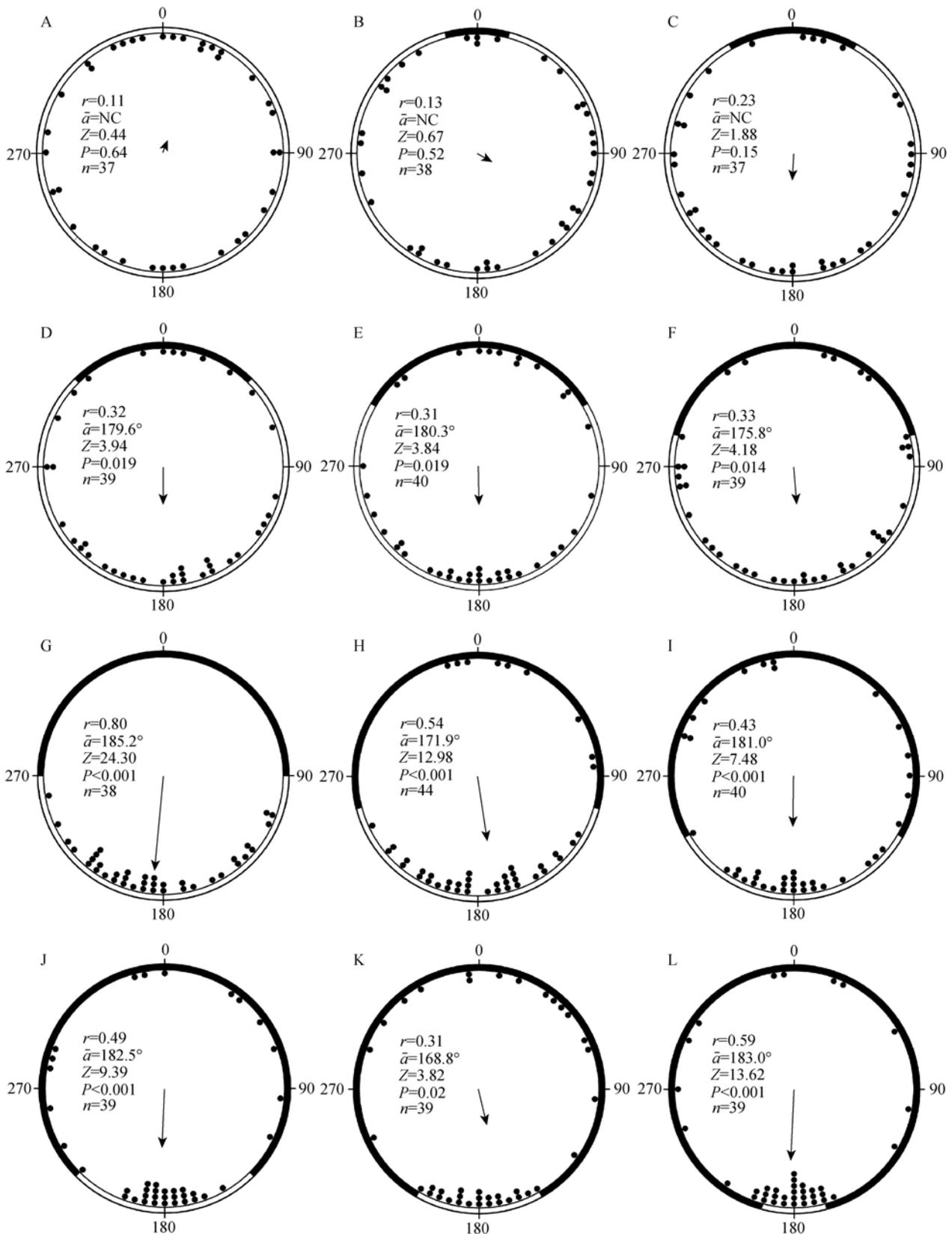


Fig. 3 Circular scatter plots of the distribution of *Limulus polyphemus* larvae in a circular arena in response to visual targets (black rectangles) ranging in size from 0° to 330° (A–L)

All trials were conducted in water containing odor from shoal grass *Halodule wrightii* (31.25 g L⁻¹). Details the same as for Fig. 1.

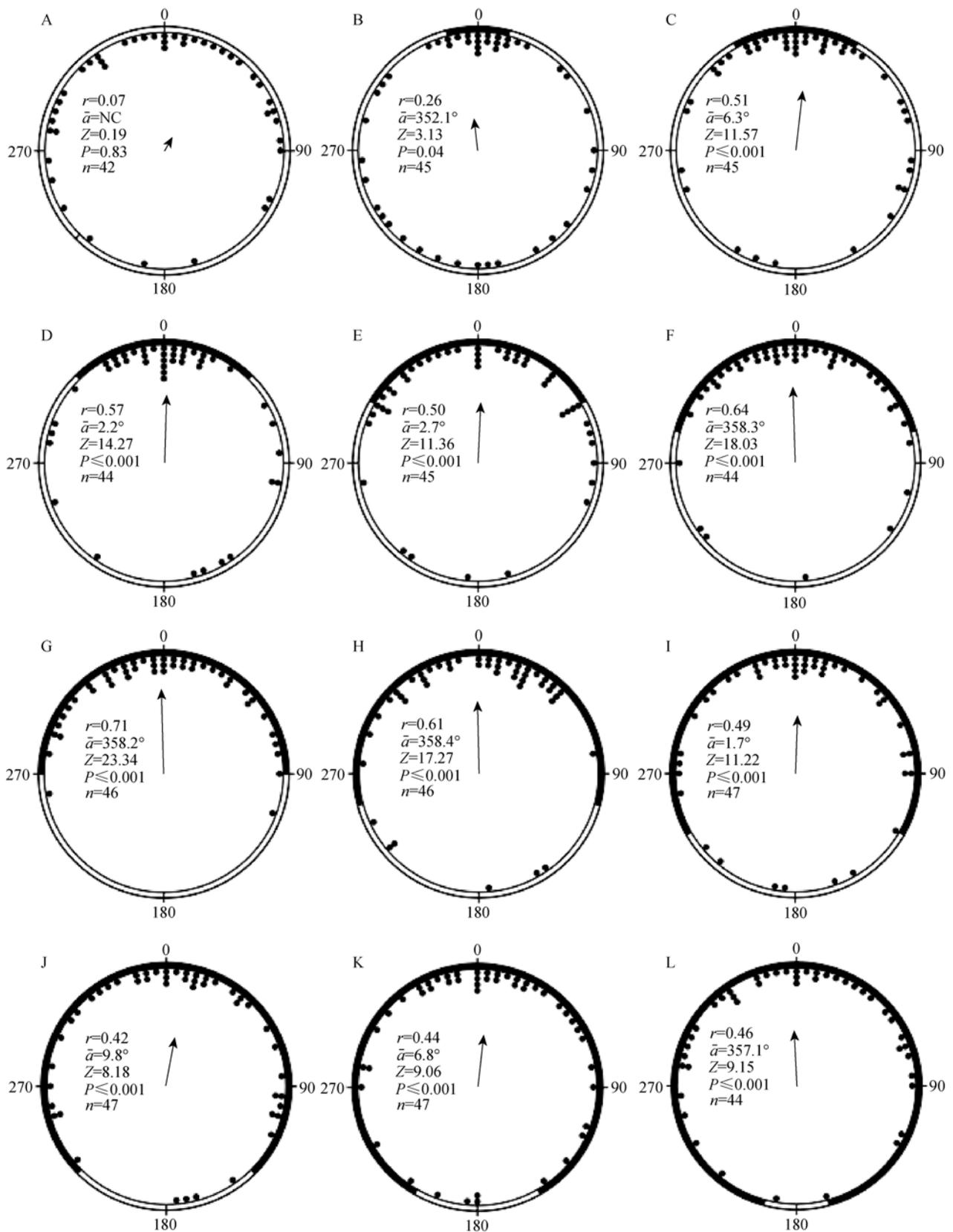


Fig. 4 Circular scatter plots of the distribution of *Limulus polyphemus* juveniles in a circular arena in response to visual targets (black rectangles) ranging in size from 0° to 330°

All trials were conducted in water containing odor from shoal grass *Halodule wrightii* (31.25 g L⁻¹). Details the same as for Fig. 1.

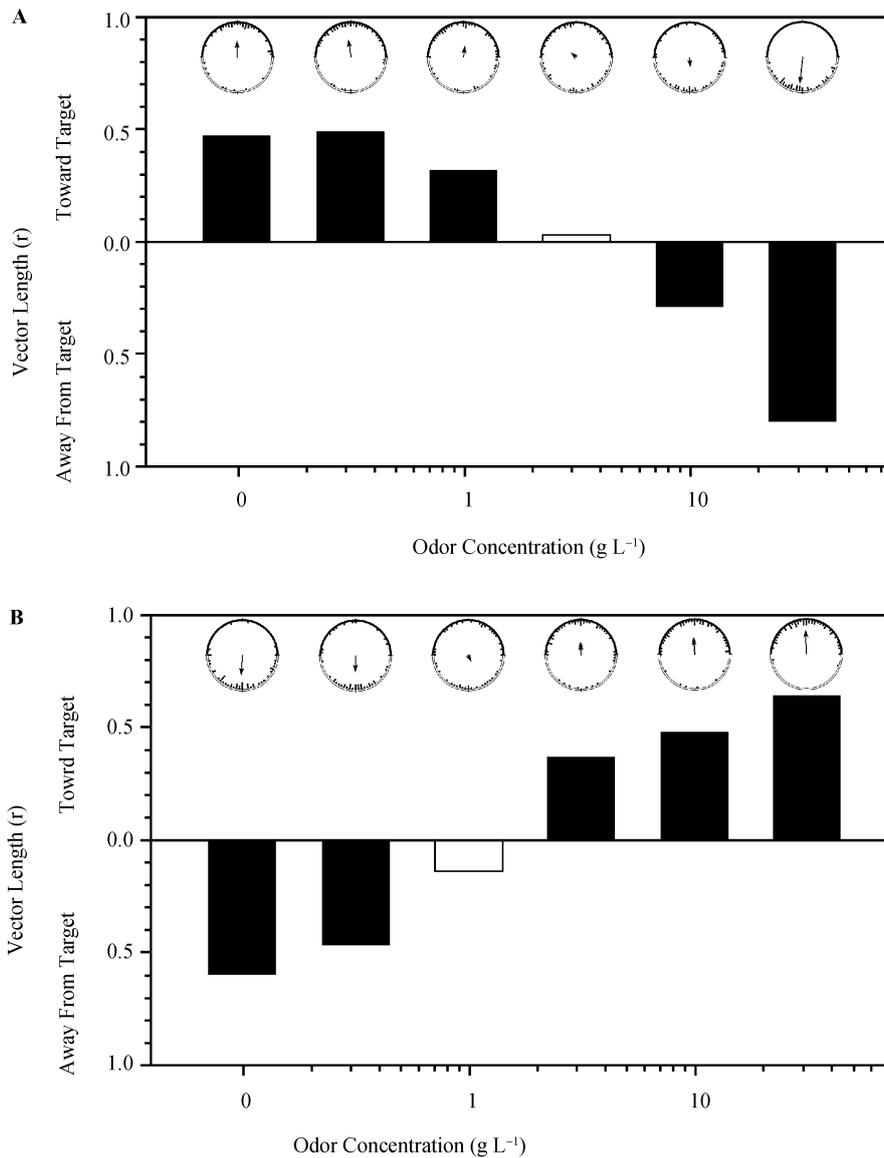


Fig. 5 Mean vector lengths (r) for *Limulus polyphemus* larvae (A) and first stage juveniles (B) in response to an 180° visual target (black rectangle) and in water that either lacked estuarine chemical cues [i.e., offshore water (control)] or contained different concentrations of chemical odor from the shoal grass *Halodule wrightii*

Vectors corresponding to mean angles directed toward and away from the target are plotted above and below the horizontal line, respectively. Circular scatter plots of the final positions of animals in the arena are plotted above each bar. Each solid dot represents the directional response of an individual crab larva or juvenile. Arrows in the centers of the scatter plots indicate the mean angles ($\bar{\alpha}$) and vector lengths (r) (radius of the circle = 1) of the distribution.

Table 2 Summary of the effects of chemical odors on the visual orientation of larval and juvenile *Limulus polyphemus* to a black rectangular target subtending an angle of 180°

	Chemical Odor Source						
	Offshore Water	<i>Halodule wrightii</i>	<i>Syringodium filliforme</i>	<i>Acanthophora</i> sp.	<i>Limulus polyphemus</i>	<i>Callinectes sapidus</i>	<i>Fundulus grandis</i>
Larvae	+	-	-	O	-	+	+
Juveniles	-	+	+	O	-	+	+

Plus (+) and minus (-) signs indicate that the direction of orientation was toward or away from the visual target, respectively. A circle (O) indicates the final position of crabs in the arena was not significantly different from uniform.

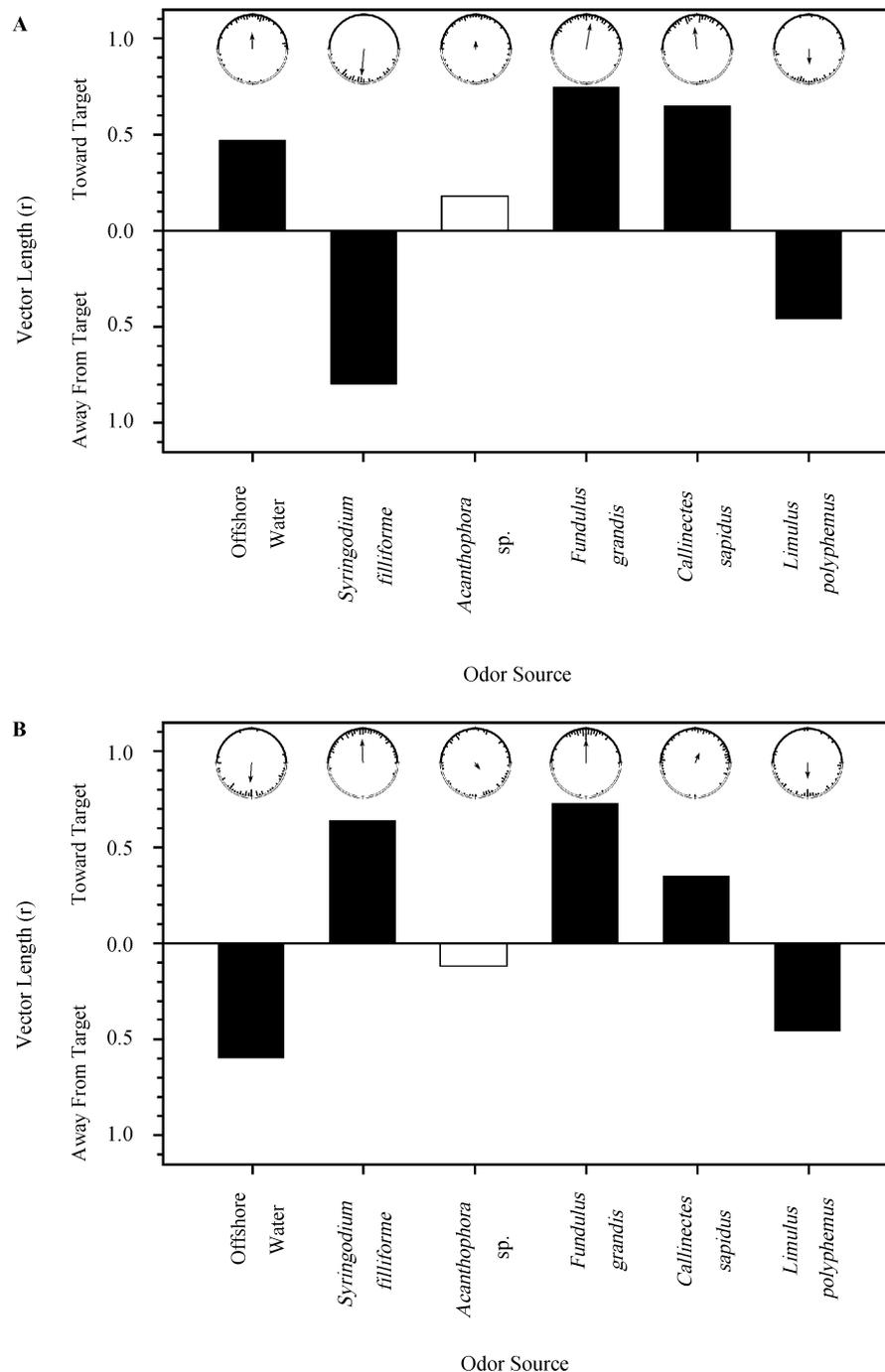


Fig. 6 Mean vector lengths (r) for *Limulus polyphemus* larvae (A) and first stage juveniles (B) in response to an 180° visual target (black rectangle) and in water that either lacked estuarine chemical cues [i.e., offshore water (control)] or contained chemical odors from potential nursery habitats (*Syringodium filliforme* and *Acanthophora* sp.), know predators (*Fundulus grandis* and *Callinectes sapidus*) or conspecifics (juvenile *Limulus polyphemus*)

Details the same as for Fig. 5.

away from the visual target ($\bar{a} = 185.2$, $r = 0.80$; $Z = 24.30$, $P < 0.001$; Fig. 6A), while juveniles oriented towards the visual target ($\bar{a} = 358.2$, $r = 0.64$; $Z = 23.34$, $P < 0.001$, $n = 46$; Fig. 6B). In contrast, when presented with odor from the drift algae *Acanthophora* sp., both

larvae and juveniles failed to respond to the visual target and their distribution in the arena was not significantly different from uniform (Larvae: $r = 0.18$; $Z = 1.31$, $P = 0.27$, $n = 39$, Fig. 6A; Juveniles: $r = 0.12$; $Z = 0.62$, $P = 0.54$, $n = 46$; Fig. 6B). Thus, movement of larvae in

water containing vegetation odors was different from their oriented response in water lacking chemical odors (i.e., offshore water; Fig. 6A) (Table 2).

In water containing odor from two known predators, the blue crab *Callinectes sapidus* and mummichug *Fundulus grandis*, larvae and juveniles responded differently to visual targets relative to the control response (Table 2). In both water types, larvae and juveniles oriented toward the visual target [(Larvae: *Fundulus grandis*: $\bar{\alpha} = 8.1^\circ$, $r = 0.75$, $Z = 22.66$, $P < 0.001$, $n = 40$; *Callinectes sapidus*: $\bar{\alpha} = 353.4^\circ$, $r = 0.65$; $Z = 13.00$, $P < 0.001$; Fig. 6A) (Juveniles: *Fundulus grandis*: $\bar{\alpha} = 0.2^\circ$, $r = 0.73$; $Z = 23.38$, $P < 0.001$, $n = 45$; *Callinectes sapidus*: $\bar{\alpha} = 21.6^\circ$, $r = 0.35$; $Z = 5.50$, $P = 0.004$, $n = 45$; Fig. 6B)]. However, because the movement of larvae was similar to their response in offshore water (i.e., control; Fig. 6A), predator odor cues only had a significant effect on the direction of orientation of juvenile crabs (Fig. 6B).

In water containing odor from conspecifics (juvenile *Limulus polyphemus*), larvae oriented away from the 180° visual target ($\bar{\alpha} = 178.7^\circ$, $r = 0.47$; $Z = 8.01$, $P < 0.001$), a response that was in the opposite direction of their orientation in offshore water (Fig. 6A; Table 2). Juveniles also oriented away from the visual target in the presence of odor from conspecifics ($\bar{\alpha} = 178.8^\circ$, $r = 0.46$; $Z = 8.84$, $P < 0.001$, Fig. 6B, Table 2), yet this response was similar to their oriented movements offshore water.

3 Discussion

The fields of optical science and visual neuroscience have benefited greatly from studies of the eye and visual system of adult *Limulus polyphemus* (reviewed by Barlow et al., 2001). However, the current study is the first detailed examination of the role of vision in the directional movements and orientation of early life history stages. Studies by Waterman (1954) and Harzsch et al. (2006) concluded that *Limulus* trilobite larvae possess rudimentary photoreceptors capable of perceiving light. This conclusion is supported by Rudloe (1979) who reported that larvae swim more actively in darkness and are positively phototactic to dim light. Shortly after hatching (~7–10 days), the lateral eyes of pre-metamorphic larvae possess numerous ommatidia, suggesting that older trilobites and post-metamorphic juveniles are capable of object recognition and directional vision (Harzsch et al., 2006). Since all trilobites used in the current study were at least 5 days post-hatching and were assumed to possess lateral eyes with functional ommatidia, we predicted that both larvae and juveniles

would be able to detect and respond to dark visual targets. We also tested the hypothesis that the direction of visual orientation changes following metamorphosis and in the presence of chemical cues from potential nursery habitats, predators, and conspecifics. An ontogenetic shift in their behavioral response was expected since larvae and juveniles occupy different habitats (water column vs. benthos) and experience different selection pressures. Both hypotheses were supported by the results.

When placed in offshore water and presented with dark, horizontal targets of varying widths, neither larval nor juvenile horseshoe crabs oriented to targets subtending angles $< 90^\circ$ (Fig. 1 A–C and Fig. 2A–C). These results suggest that targets of these sizes are not large enough to be detected or elicit a response. Trilobite larvae were attracted to larger targets subtending angles $\geq 90^\circ$ and their distribution became more concentrated with an increase in target size (Fig. 1D–L). Yet, following metamorphosis, juvenile crabs moved in the opposite direction in response to the same visual cues (Fig. 2D–L). Errigo et al. (2001) and Ridings et al. (2002) reported a similar avoidance of dark, high-contrast objects by older juvenile *L. polyphemus*. The percentage of both life history stages exhibiting oriented movements (either toward or away) to targets was high (range: 66%–96%), yet juveniles were typically more responsive than larvae to the same size target (Table 1). Response rates may be a consequence of differences in the size, number, or sensitivity of the ommatidia. Although the photosensitivity and acuity of the lateral eyes of larval and newly metamorphosed juvenile *L. polyphemus* is unknown, the visual field of post-hatch crabs is expected to increase with development since the number of ommatidia increases from a ~17 in late trilobite larvae, to ~30 in first stage juveniles, and ~1000 in adults (Waterman, 1954; Meadors et al., 2001; Harzsch et al., 2006).

The second part of the study involved monitoring changes in orientation to visual targets in the presence of chemical cues (i.e., chemically mediated visual orientation). Although horseshoe crabs are known to possess a variety of chemoreceptors (Patten, 1894; Barber, 1961; Hayes, 1966, 1971; Hayes and Waterman, 1969; Wyse, 1971) and chemical cues have been shown to be used by adult *Limulus* for finding food and mates (Wyse, 1971; Hassler and Brockmann, 2001; see Saunders et al., 2010 for review), the olfactory capabilities and chemoreceptive responses of larval and juvenile crabs have not been documented. Since juvenile crabs are known to

inhabit sandflats as well as vegetative areas dominated by macroalgae and seagrass following metamorphosis (Shuster, 1979; Tankersley and Boleman, unpublished data), dark rectangles were expected to represent habitat and it was predicted that, in the presence of odors from benthic vegetation, planktonic trilobite larvae would avoid these areas and juvenile crabs would be attracted to targets. This hypothesis was supported since larvae oriented away from visual targets in water containing odor from the seagrasses *Halodule wrightii* and *Syringodium filliforme* (Figs. 3D–L, 6A; Table 2). Orientation away from visual targets in the presence of a chemical cue can be interpreted in two ways: as a reaction to the visual target or a phototactic response to light. As suggested by Rudloe (1979), larvae may use positive phototaxis and avoidance of dark areas to move toward the surface and emerge from the nest following hatching. Once in the water column, upward movement towards light by *Limulus* larvae may contribute to position maintenance in the water column and aid in dispersal and transport away from the beach and shallow near-shore areas.

As with larvae, the direction of visual orientation of post-metamorphic juvenile *L. polyphemus* changed in the presence of chemical odors from seagrasses. When placed in water containing odors from *H. wrightii* and *S. filliforme*, juveniles moved toward dark targets (Figs. 4D–L, 6B; Table 2). This behavior was interpreted as a shelter-seeking response. In the presence of seagrass odors, juvenile crabs may interpret a dark horizon as habitat and move toward it. Similar “attractive chemicals” have been found to trigger shelter- and host-seeking orientation behaviors in the snapping shrimp *Synalpheus demani* (Huang et al., 2005) and the hermit crabs *Clibanarius vittatus* (Hazlett, 1982; Diaz et al., 1995a,b) and *C. antillensis* (Chiussi et al., 2001). Additional studies are needed to determine whether similar chemical cues elicit other oriented behaviors (e.g., chemotaxis, chemically mediated rheotaxis) in post-metamorphic juvenile horseshoe crabs that could be involved in habitat selection.

Ontogenetic changes in chemically mediated visual orientation in the presence of habitat associated chemical cues have been reported in other marine arthropods, most notably megalopae and juveniles of the blue crab *Callinectes sapidus* (Diaz et al., 1999, 2001, 2003). Intermolt megalopae exhibit an escape or predator avoidance response and orient away from solid and vertical stripe targets in the presence of odors from seagrass and predators (Diaz et al., 1999). As postlarvae approach

metamorphosis, this avoidance behavior changes in predator odor water. Megalopae remain motionless or swim away from solid objects and swim in all directions when presented with vertical stripes. The swimming orientation of premolt megalopae in flow (upstream vs. downstream) also changes in the presence of odors from potential predators (Forward et al., 2003). Post-metamorphic juvenile blue crabs also swim away from solid targets in the absence of habitat chemical cues but remain immobile or swim haphazardly in the presence of cues for aquatic vegetation and predators. This alarm/fright response is not exhibited in later stage crabs (Stage IV and V) (Diaz et al., 2001, 2003).

The effect of chemical odors from *H. wrightii* on the visual orientation of crabs was concentration-dependent. Larvae and juveniles showed a directed response which reversed as the concentration of *H-w* odor decreased (Fig. 5A,B). Orientation away from the visual target (response to 31.3 g L⁻¹ *H-w* odor water) ceased when the concentration reached 3.13 g L⁻¹. At this concentration, the distribution of larvae around the arena was not significantly different from a uniform distribution (Fig. 5A). At a concentration of 0.98 g L⁻¹, larvae no longer responded significantly to *H-w* odor (Fig. 5A). Similarly, juvenile crabs ceased moving toward the visual target and their distribution in the arena became uniform at a concentration of 0.988 g L⁻¹. At seagrass concentrations \leq 0.31 g L⁻¹, orientation became more directed and the distribution of juveniles in the arena was similar to their response in offshore water (i.e., away from the visual target; Fig. 5B). This concentration was 30% less than the threshold for larvae. Observed differences in sensitivity may be due to the development of the nervous system and associated sensory structures involved in chemoreception. However, the relationship between *H-w* odor concentration and orientation direction suggests that behaviors and responses of crabs to visual cues changes with their proximity to different estuarine habitats. Thus, as juveniles encounter chemical odors from seagrass, they orient toward dark areas and their movements become more directed as they get closer and the concentration increases. Under the same scenario, planktonic larvae move upward (positive phototaxis) and away from dark areas.

When placed in water containing odor from drift algae *Acanthophora* sp., larvae and juveniles failed to orient to the target and were distributed uniformly around the arena (Fig 6A, B). The response was not expected since juvenile horseshoe crabs are known to inhabit clumps of *Acanthophora* sp. following meta-

morphosis (Tankersley and Boleman, unpublished data). Nevertheless, this behavioral pattern (i.e., movement in all directions) is often interpreted as an alarm response (Diaz et al., 2001) and may be attributed to noxious chemicals extruded by the algae. Chemicals extracted from *Acanthophora muscoides* are known to be toxic to terrestrial arthropods (mosquitoes) (Prabha Devi et al., 1997). Similar chemicals may have leached-out of the *Acanthophora* sp. during the preparation of odor water and negatively affected the crabs.

In water containing odors from two known predators, *Fundulus grandis* and *Callinectes sapidus*, larval horseshoe crabs oriented toward the visual target (Fig. 6A; Table 2). This response did not differ from the control response in offshore water, indicating that these odors had no apparent effect on orientation. However, in similar predator water, the orientation of juveniles changed dramatically and was directed towards the visual target (Fig. 6B; Table 2). Since black rectangles of different sizes were expected to represent predators, we predicted that juvenile crabs would avoid the targets in the presence of predator odors (escape response; e.g., Lima and Dill, 1990; Atema, 1995). This hypothesis was not supported and the oriented movements of juvenile crabs were more consistent with a shelter-seeking behavior than a predator avoidance response.

Larvae responded to conspecific odor by orienting away from the visual target. This direction of orientation was the opposite of what was observed in offshore water. Conversely, juveniles did not react to the odor of conspecifics as their responses were similar to those exhibited in offshore water. These results were unexpected and inconsistent with those observed in other arthropod species. In general, arthropods typically respond to conspecific odor by moving towards the source of the chemical cue, increasing settlement and aggregation in larvae (review by Burke, 1986). The lack a response to conspecific odor suggests that both pre-and post-metamorphic *L. polyphemus* are not gregarious and do not seek shelter or settlement habitats occupied by other horseshoe crabs.

Results of the current study indicate that horseshoe crab larvae and juveniles are capable of detecting and responding to visual and chemical cues and are also able to combine information from two sensory modalities to respond in a manner that would be beneficial to their survival. Larval and juvenile horseshoe crabs live in different environments (pelagic vs. benthic) and experience different selective pressures and challenges. Moreover, changes in their sensory capacities, espe-

cially their optical and chemosensory systems, following metamorphosis likely increases their ability to detect and respond to visual and chemical cues. Ontogenetic shifts in visual orientation have been reported in a variety of arthropod species (e.g., Welsh, 1932; Christensen and McDermott, 1958; Thorson, 1964) and these results give additional support to the hypothesis that the nature of the responses to both olfactory and visual stimuli may change throughout development due to changes in lifestyle, habitat, and physiology.

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