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EFFECTS OF DIFLUBENZURON ON THE ONTOGENY OF PHOTOTAXIS BY *PALAEMONETES PUGIO*

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ABSTRACT The phototaxis by larvae of the grass shrimp *Palaemonetes pugio* that hatched from embryos which were exposed to a single pulse concentration of diflubenzuron (DFB; Dimilin®) was quantified. Stage IV embryos (6-day-old) were exposed to 0.5 µg/L of DFB for 4 days followed by transfer into clean seawater for the rest of the incubation period. The photoresponses of light-adapted larvae from untreated embryos and embryos treated with 0.5 µg/L DFB were monitored from 1 day through 8 day post hatch for phototactic responses to 500 nm light. Larvae from untreated embryos exhibited strong positive phototaxis at high light intensities (3×10^{-2} and 3×10^{-1} Wm^{-2}) but became negatively phototactic at lower light intensities (3×10^{-5} to 3×10^{-3} Wm^{-2}). This phototactic pattern continued during the monitoring period. On the other hand, larvae from DFB-treated embryos exhibited altered phototaxis for the first 3 days. Alterations were especially evident on Day 1, as larvae were only negatively phototactic. By Day 4, these larvae reverted to the normal pattern of photoresponses shown by untreated larvae. These results indicated that the alterations in photoresponses of larvae caused by embryonic exposure to DFB are only transitory and can be corrected within 4 days of hatching if the larvae are exposed to water lacking DFB.

Introduction

Diflubenzuron (DFB; Dimilin®) is an insect growth regulator that interferes with chitin formation and molting in arthropods. It is approved for and is being used in the United States for control of a wide variety of insect pests, including foliage feeders on soybeans, cotton-leaf perforator, and forest insects. In California DFB is used to control mosquito larvae (Fischer and Lenwood 1992). The effects of DFB on non-target anthropods, especially aquatic organisms, is well documented (see review by Fischer and Lenwood 1992). There is always the potential for DFB impacting aquatic organisms because of overspray or spills, especially where it is being applied close to water or directly onto wetlands for mosquito control.

Phototaxis and its ecological significance in crustaceans is well documented in the literature (White 1924, Thorson 1964, Forward 1974, Vernberg et al. 1974, Forward et al. 1984, Sulkin 1984). For example, in a review by Thorson (1964) of marine benthic invertebrates, of the 141 species studied, 82% of the early larval stages respond positively to light. Phototaxis has also been reported to play an important role in diel vertical migration of crustacean larvae (Forward 1976, Forward and Cronin 1980, Forward et al. 1984, Forward 1985). Vertical migration contributes to the dispersal of crustacean larvae and helps in their retention in the estuary (Sulkin 1975, Cronin 1979, Cronin and Forward 1986).

For larval stages of estuarine crustaceans, the phototactic pattern, when tested in a narrow light field, is generally negative phototaxis to low light intensities and positive phototaxis to moderate intensities (e.g. Forward and Costlow 1974). Also, ontogenetic changes in photoresponses are observed in some crustaceans. Generally, the younger stages are more positively phototactic while negative phototaxis increases in the older larval stages, postlarvae, and adults (see review by Pardi and Papi 1961, Dingle 1969). Because of the role of phototaxis in vertical migration of crustacean larvae, any alteration in this photoresponse as a result of exposure to toxicants may affect the ecology and conceivably the larvae's recruitment into the adult population.

Photo behavior has been shown to be very sensitive to changes in environmental factors such as temperature, salinity, and chemicals. Changes in photobehavior have also been used in aquatic toxicology as a sensitive indicator of anthropogenic stress (Rosenthal and Alderdice 1976, Simonet et al. 1978, Lang et al. 1981, Rand 1985). Specifically for larval crustaceans, the following studies have employed changes in photobehavior as indicators of sublethal toxicity: Forward and Costlow (1976) for insect juvenile hormone mimic on *Rhithropanopeus harrisii*; Moyer and Barthalmus (1979) for the herbicide Weeder-64 on *Palaemonetes pugio*; Lang et al. (1980) for copper on *Balanus improvisus*. In all these studies, the larvae were directly exposed to the toxicant followed by measurement of phototaxis. Only Wilson (1985) and Wilson et al. (1985) have reported alterations in phototaxis by larval stages of crustaceans as a result of embryonic exposure to a toxicant. Both the level and sign of phototaxis were altered in light-adapted first stage larvae of *P. pugio* after 4-day single pulse exposure of the embryos to DFB. These alterations in phototaxis were shown to be dependent on the DFB concentration and the embryonic stage at exposure

(Wilson et al. 1985). The present study was conducted to determine if and when larval grass shrimp from DFB-treated embryos which exhibit altered phototaxis regain normal pattern of phototaxis during larval development.

MATERIALS AND METHODS

Ovigerous female grass shrimp *P. pugio* that were induced to spawn in the laboratory (Duke University Marine Laboratory, Beaufort, NC) were sorted according to stage of embryonic development as described by Wilson (1985). Laboratory animals were used in this study because they were relatively homogeneous and gave less variable results than field animals. Only ovigerous females carrying Stage IV embryos (6-day-old; body segmentation stage, at 25 ± 1°C) were used in this study. Earlier studies by Wilson (1985) and Wilson et al. (1985) have shown that Stage IV embryos are the most sensitive embryonic stage and represent a midpoint in the embryonic development of *P. pugio*. The shrimp were placed in large culture dishes (inside diameter = 20 cm) containing 0.5 µg/L of wettable powder (WP-25%) formation of DFB dissolved in 20‰ filtered (to 45 µm) seawater. Untreated 20‰ filtered seawater served as the control. This test concentration was used because Wilson et al. (1985) have shown that for phototaxis, 0.5 µg/L is the lowest observed effect concentration (LOEC) when various embryonic exposure concentrations were used. The shrimp were maintained at a density of 5 per liter of test solution for 4 days without renewal (single dose exposure). After the 4-day exposure, the shrimp were transferred into clean seawater (20‰), which was changed every day until the eggs hatched. The larvae were then used in phototaxis experiments. The rationale for exposing embryos rather than larvae is that this test protocol, delayed sublethal bioassay (DSB), has been shown to be more sensitive than shrimp or crab larval bioassays (see Wilson 1985 for details). Ovigerous females and larvae were reared in an environmental chamber set at 25°C and 12L:12D photoperiod, centered at 1200 h. Animals were fed freshly hatched *Artemia* sp. nauplii daily.

Experiments were performed to determine ontogeny of phototaxis of larvae hatched from unexposed embryos (control) and embryos exposed to 0.5 µg/L DFB. The general protocol for all phototaxis experiments was that described by Wilson et al. (1985) with few modifications. Phototaxis was determined by measuring the direction of swimming immediately following light stimulation. Ten to 15 larvae were placed in an acrylic trough measuring 14.9 x 8.3 x 3.5 cm containing approximately 110 ml filtered seawater (20‰). The trough was divided into 5 equal compartments by acrylic partitions which could be raised or lowered simultaneously. The stimulus light was presented horizontally from a slide projector fitted with a 300 watt incandescent bulb. The light was interference-filtered to 500 nm (7 nm halfbandwidth). This wavelength was selected because it has been shown to be the spectral sensitivity maximum for *P. pugio* (personal communication, John K. Douglas, University of Arizona, Tucson, AZ 85721, unpublished) and *P. vulgaris* (White 1924). Intensity was regulated by neutral-density filters (Detric Optics, Inc.) and measured with a radiometer (from EG&G model 550).

Phototaxis measurements were performed in a photographic darkroom between midnight and 0300 h. This time was chosen to coincide with the time of maximum larval release by laboratory-maintained ovigerous females (personal observations), thereby ensuring that larvae were 24 ± 2 h old when first tested. By monitoring phototaxis at the same time of day for all experiments, complications due to biological rhythms in behavior (see Forward and Cronin 1980) were avoided. Shrimp larvae were light adapted for 4 -6 h to 12.53 Wm^{-2} light intensity (cool-white fluorescent lamps) prior to testing. Ten to 15 larvae were placed in the central compartment of the acrylic trough and allowed to adapt in darkness for 30 s. After this, the partitions were raised gently and the stimulus light turned on simultaneously. Larvae were then stimulated for 60 s then the partitions were lowered and the stimulus light turned off. The number of larvae in each compartment was recorded. Larvae were returned to rearing conditions and tested on subsequent days. A new group of larvae were then introduced into the trough and tested as previously outlined. This procedure was repeated at least 3 times before the neutral density filters were changed to test a different intensity of the stimulus light. Six to 7 different light intensities were tested plus a "dark control" in which the movements of larvae in the test trough were monitored without any stimulus light. Different larvae were used for each stimulus light level. The larvae were fed throughout the phototaxis experiments to reduce the possibility of altered phototaxis due to starvation (Cronin and Forward 1980, Lang et al. 1980). The intensity versus response curves for these larvae were again determined on the second day (i.e., for 2-day-old larvae). Using the same batch of larvae, this procedure was repeated every day up to Day 4 and again on Day 8. Examination of both untreated and treated larvae on Day 4 indicated that they had stalked eyes and thus had molted to the 2nd zoeal stage.

Positive phototaxis was defined as movement towards the light source and negative phototaxis as movement away from the light source. The animals in the 2

compartments closest to the light source were regarded as showing positive phototaxis; those in the 2 compartments farthest from the light source as negatively phototactic. The mean percentage positive and negative response and their standard errors (S.E.) were calculated at each light intensity. For statistical analysis, the percentages were first arcsine transformed. Statistical tests determined the difference between dark control (no light stimulus) response levels due to movement in the test trough in darkness and responses upon stimulation with light. Chi-square tests and analysis of variance were performed on the results as described by Sokal and Rohlf (1981). The level of significance was set at $P = 0.05$ for all tests.

RESULTS

Larvae from Unexposed Embryos

The intensity versus response curves for light-adapted larvae from unexposed embryos during ontogeny are shown (Figure 1). The pattern of phototaxis exhibited by Day 1 larvae (Stage 1) remains virtually the same through Day 8 of development. As compared to the dark control level of responsiveness, larvae were positively phototactic ($P < 0.05$; ANOVA) at the stimulation intensity of 3×10^{-1} (days 2, 4, and 8) or at 3×10^{-2} Wm^{-2} and higher intensities (Days 1 and 3). Larvae were negatively phototactic ($P < 0.05$; ANOVA) at lower light intensities with the threshold being 3×10^{-5} Wm^{-1} for Days 1 to 4 and one log unit higher for Day 8.

There is some indication of increased activity by the larvae with age as evidenced by the increase in the dark control responses of larvae. The positive control (no light present) increased from 26% on Day 1 to 40% on day 8 (Figure 1).

Larvae from Embryos Exposed to DFB

The ontogenetic changes observed in the photoresponses of light-adapted larvae that hatched from embryos (Stage IV) exposed to 0.5 µg/L DBF are presented in Figure 2. Positive phototaxis was absent (relative to the dark controls) at the stimulation intensities that normally evoked significant positive responses in untreated larvae (3×10^{-2} Wm^{-2} and higher; Figure 1). Compared with Day 1 untreated larvae (Figure 1), the larvae from DFB-treated embryos exhibited negative phototaxis ($P < 0.05$; ANOVA) (Figure 2) over a much wider range of stimulus intensities (3×10^{-5} to 10^{-1} Wm^{-2}).

By Day 2, the first sign of a return to the normal pattern of phototaxis was evident as seen by an increase in positive phototaxis from the control level on Day 1 to 72% on the second day at 3×10^{-1} Wm^{-2} stimulation intensity (Figure 2). The positive responses at 3×10^{-1} Wm^{-2} on Days 2 and 3 by treated larvae are not significantly different ($P > 0.05$; chi-square) from each other (Figure 2). At an intensity of 3×10^{-2} Wm^{-2}, Days 2 and 3 larvae remained strongly negatively phototactic. However, by Day 4, the larvae exhibited positive phototaxis at both 3×10^{-2} and 3×10^{-1} Wm^{-2} (see Figure 2). Thus, the return to normal photoresponse is complete by Day 4 for larvae from embryos exposed to 0.5 µg/L DFB. The response patterns exhibited by 4- and 8-day-old larvae were almost identical. The lowest light intensity evoking positive phototaxis and the highest intensity that evokes negative phototaxis for unexposed and exposed larvae are compared in Table 1. Although these threshold intensities were very different for 1-day-old treated and untreated larvae, they became identical by Day 4.

DISCUSSION

The phototactic pattern of Stage I larvae from the grass shrimp *P. pugio* has been extensively documented by Wilson (1985) and Wilson et al. (1985). The pattern of phototaxis of light adapted Stage I larvae from untreated embryos was positive phototaxis at high light intensities (3×10^{-2} and 3×10^{-1} Wm^{-2}) and negative phototaxis at lower light intensities (3×10^{-5} to 3×10^{-2} Wm^{-2}; Figure 1; Wilson et al. 1985). This pattern of phototaxis persists for larvae from untreated embryos irrespective of the age of the embryos when incubation started in the laboratory (Wilson 1985, Wilson et al. 1985). For larvae that hatched from DFB-treated embryos, both the magnitude and the sign of the photoresponse were altered. Such larvae consistently exhibited negative phototaxis at higher light intensities that normally evoke positive phototaxis (3×10^{-2} and 3×10^{-1} Wm^{-2}). These alterations in phototaxis varied upon exposing embryos to concentration of DFB ranging from 0.3 to 1.0 µg/L (Wilson et al. 1985). However, at exposure concentrations of ≥ 2.5 µg/L, larvae exhibited severe structural abnormalities, and the magnitude of both positive and negative phototaxis was drastically reduced (Wilson 1985).

Results of the present study indicate that for light-adapted Stage I larvae from unexposed embryos, phototaxis remains virtually unchanged during larval development. Both the pattern of the stimulus light intensity versus phototactic response curves and the magnitude of the phototactic responses were similar for all the larval stages tested (up to 8 days old). It should be pointed out that this pattern of phototaxis by light-adapted larvae was also observed up to Day 15 (Wilson unpublished data). However, at the postlarval stage (unpublished data) both positive and negative phototaxis are lost since the animals

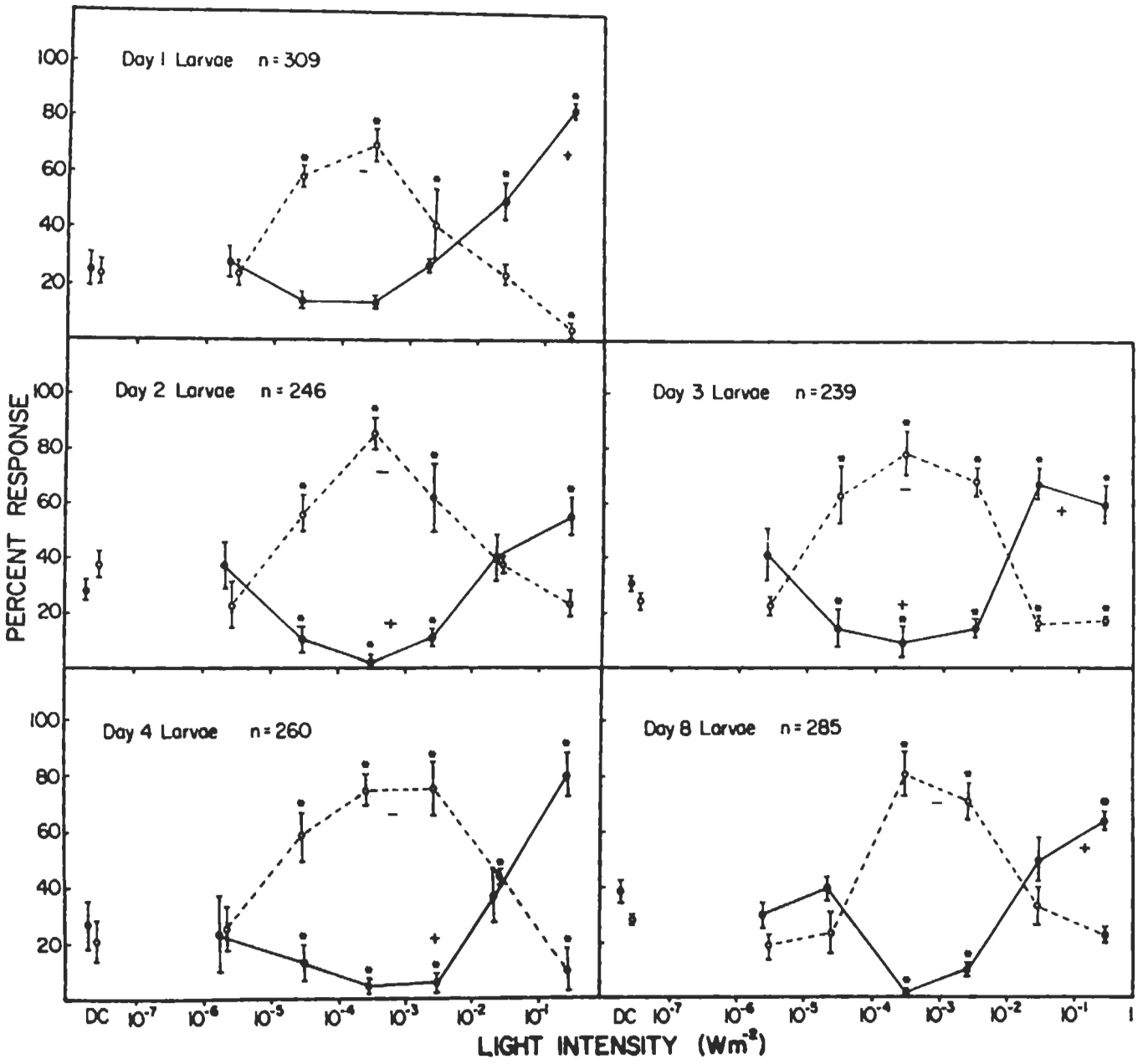

Figure 1. *Palaemonetes pugio.* Intensity versus response curves for different ages of light-adapted larvae hatched from untreated embryos (i.e., incubated in seawater throughout embryonic development). Open circles, dashed lines represent negative phototaxis. Closed circles, solid lines represent positive phototaxis. DC = dark control values for larvae moving to the positive and negative chambers of the test trough in the absence of light. Data points are means ± S.E. The sample size (n) for each stimulus intensity was 3. Asterisks indicate means that are significantly ($P < 0.05$) greater or less than the appropriate dark control. Embryos were 6 days old when incubation started.

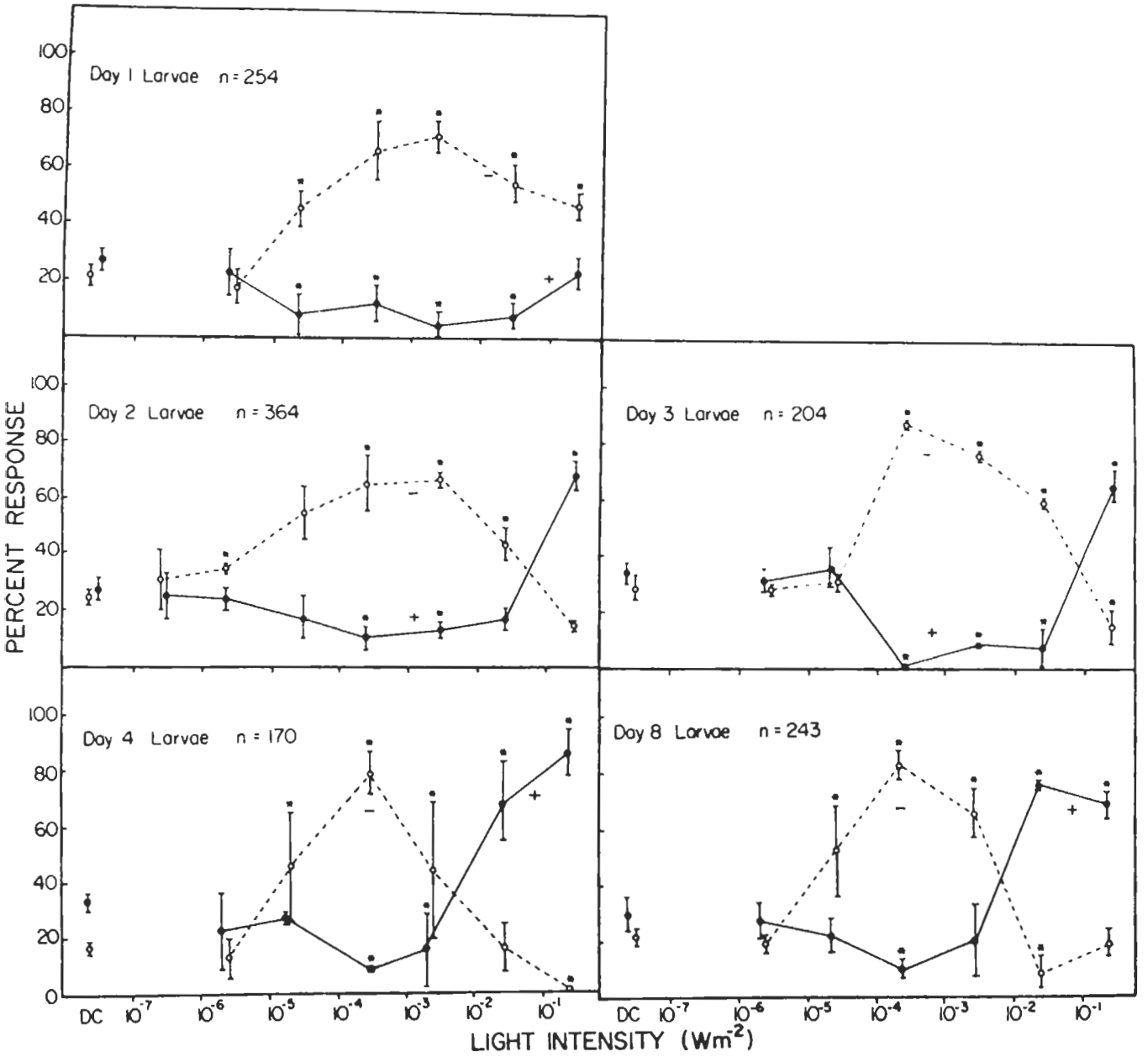

Figure 2. ***Palaemonetes pugio.*** **Intensity versus response curves for different ages of light-adapted larvae hatched from embryos that were exposed to 0.5 µg/L diflubenzuron starting when they were 6 days old. Open circles, dashed lines represent negative phototaxis. Closed circles, solid lines represent positive phototaxis. DC = dark control values for larvae moving to the positive and negative chambers of the test trough in the absence of light. Data points are means ± S.E. The sample size (n) for each light intensity was 3. Asterisks indicate means that are significantly ($P < 0.05$) greater or less than the appropriate dark control.**

TABLE 1

Comparison of lowest light intensity that evokes positive phototaxis and highest light intensity evoking negative phototaxis in grass shrimp larvae from untreated control and diflubenzuron (DBF)-exposed embryos. NR is no phototactic response.

Larval Age (Days)	Positive Response (Lowest Intensity) Wm^{-2}		Negative Response (Highest Intensity) Wm^{-2}	
	untreated	DFB-exposed	untreated	DFB-exposed
1	$3x10^{-2}$	NR	$3x10^{-3}$	$3x10^{-1}$
2	$3x10^{-2}$	$3x10^{-1}$	$3x10^{-3}$	$3x10^{-2}$
3	$3x10^{-2}$	$3x10^{-1}$	$3x10^{-3}$	$3x10^{-2}$
4	$\mathbf{3x10^{-2}}$	$\mathbf{3x10^{-2}}$	$\mathbf{3x10^{-3}}$	$\mathbf{3x10^{-3}}$
8	$3x10^{-2}$	$3x10^{-2}$	$3x10^{-3}$	$3x10^{-3}$

were unresponsive to even the highest stimulation intensity used (3×10^{-1} Wm^{-2} at 500 nm light). Forward and Costlow (1974) have reported a similar pattern in phototaxis during ontogeny for the mud crab, *R. harrisii*. Both the action spectra and the intensity versus response curves for light- and dark-adapted animals were similar for all zoeal stages. On metamorphosis into the megalopa stage, there was a dramatic change in behavior similar to that reported here for the postlarvae of the grass shrimp. These findings are different from those reported by Welsh (1932) for the mussel crab and by Hunte and Myers (1984) for estuarine amphipods, where changes from positive to negative phototaxis were observed during larval development. In some instances, (e.g. in *Balanus)* there is a change from positive phototaxis in newly hatched nauplii to negative in Stage II and back to positive in the cyprid stage (Thorson 1964).

The lack of ontogenetic changes in phototaxis of *P. pugio* larvae from untreated embryos made it relatively easy to determine when larvae from DFB-treated embryos regained normal photobehavior. By comparing the pattern of the intensity versus response curves for each age of the larvae from untreated and DFB-treated embryos, it was observed that a return to normal photobehavior started with Day 2 larvae and by the time they were 4 days old, the response patterns were similar to that of the untreated group. Thus, it is possible for larvae with altered photobehavior resulting from embryotoxicity of DBF to regain their normal photoresponsiveness within 2 to 4 days if reared in clean seawater during larval development.

Microscopic examination indicated that 4-day-old treated and untreated larvae had molted to the 2nd zoeal stage in the present experiment. Therefore, the change back to normal pattern of phototaxis by light-adapted larvae from DFB-exposed embryos was completed after the larvae molted to the 2nd stage. Although there are reports of altered phototaxis by crustacean larvae and adults resulting from exposure to toxicant (Bigford 1977, Forward and Costlow 1976, Lang et al. 1980, Moyer and Barthalmus 1979, Wilson et al. 1985), the present study is the first report of re-establishment of normal phototaxis upon removal of the toxicant during larval development.

In untreated Stage I larvae the eyes are sessile with cuticular lens and apposition optics, i.e., the lenses form small inverted images on the rhabdoms (Land 1984, Fincham 1984). For details on the structure and function of grass shrimp eyes, see Parker (1897), Douglass (1986), and Douglass and Forward (1989). Ontogenetic study of the compound eyes of *P. pugio* from larval to postlarval stage shows that the basic morphological and anatomical organization of the eyes remain unchanged throughout larval development (Douglass and Forward 1989). It is therefore not surprising that the photoresponse of untreated larvae remain the same during larval development in this study. The altered photoresponse seen in larvae from DFB-exposed embryos is conceivably the result of structural modification of the visual system of the larvae. Grass shrimp larvae hatched from embryos exposed to 0.5 µg/L DFB have been shown to exhibit slight morphological abnormalities (terata), which also affect swimming speed and vertical distribution in a seawater column (Wilson et al. 1985, Wilson et al. 1987).

Ultrastructural study of the exoskeleton of the mud crab *R. harrisii* by Christiansen and Costlow (1982) revealed that larvae exposed to DFB had disorganized and swollen exocuticle. Since the thickness of the cuticle is the same in *Rhithropanopeus* and *Palaemonetes* (Freeman 1993) and the effects of DFB on larval crustaceans is similar, it can be presumed that larvae from DFB-treated embryos may have swollen and malformed cuticular facets in the eyes. Such swollen cuticular facets may alter the entire optics of the larval eyes and could account for the

observed reversal in phototaxis. In apposition eyes, the cuticular facet acts as a lens which focuses light on the rhabdom (Cronin 1986). Conceivably, when the lens is not properly formed, e.g., has granular disorganized endocuticle (see Mulder and Gijswijt 1973), or is swollen, the amount of light passing through will be reduced. This may explain why exposed larvae responded negatively at light intensities to which they normally reacted positively. Normal phototaxis is restored upon molting probably as a result of formation of new cuticular facets with normal thickness and endocuticle. It is also possible that the distribution of the visual pigments in DFB-treated larvae is altered as a result of biochemical changes. Irrespective of what mechanism caused alteration in phototaxis, it is clear from the present study that normal phototaxis was restored after the larvae molt to the 2nd zoeal stage.

Since larvae were tested in an unnatural light field (e.g. Forward 1985), relating phototaxis to actual behavior in nature is difficult. Nevertheless, the results do indicate photobehavior was altered by exposure to DFB, and thus, aspects of larval ecology that depend on photobehavior would be altered. Photobehavior is involved in diel vertical migration of the larvae, and hence their temporal vertical distribution in an estuary (Allen and Barker 1985) could be altered. Since their vertical distribution affects horizontal transport, recruitment to the adult population would be affected. The ability to avoid predators could also be reduced by alterations in photobehavior, since the negative phototaxis participates in a predator avoidance shadow response (Forward 1977). Also, Douglass et al. (1992) demonstrated that *P. pugio* larvae have endogenous phototaxis rhythm, which if altered would change the photoresponse pattern throughout the tidal cycle in an the estuary. Thus, the survival potential of the shrimp population could be reduced by alteration in larval photobehavior.

In summary, the pattern of phototaxis by grass shrimp larvae from untreated embryos remains unchanged during larval development. This pattern consists of a positive phototaxis at high light intensity ($\geq 3 \times 10^{-2}$ Wm^{-2}) and negative phototaxis at lower intensities ($\leq 3 \times 10^{-3}$ Wm^{-2}). Although larvae from DFB-treated embryos had altered phototaxis, photobehavior was gradually restored as the larvae developed in clean water, and restoration was complete upon molting to the 2nd zoeal stage. Hence, altered phototaxis as a result of embryotoxicity to DFB is only temporary in grass shrimp larvae.

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