

Sensitivity of Crayfish *Procambarus clarkii* Antennules to Hydrodynamic Stimuli is Reduced during Diazinon Exposure

Harold M. Monteclaro¹, Kazuhiko Anraku^{1,*}, Seiichi Uno¹, Jiro Koyama¹, Tatsuro Matsuoka¹, and Hong-Young Yan²

¹Faculty of Fisheries, Kagoshima University, Shimoarata 4-50-20, Kagoshima City 890-0056, Japan

²Marine Research Station, Institute of Cellular and Organismic Biology, Academia Sinica, Jiaoshi, Ilan County 262, Taiwan

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Harold M. Monteclaro, Kazuhiko Anraku, Seiichi Uno, Jiro Koyama, Tatsuro Matsuoka, and Hong-Young Yan (2011) Sensitivity of crayfish *Procambarus clarkii* antennules to hydrodynamic stimuli is reduced during diazinon exposure. *Zoological Studies* 50(2): 145-154. In this study, the susceptibility of crustacean antennular mechanosensitivity to a pesticide was examined. We investigated the sensitivity of crayfish *Procambarus clarkii* Girard antennules to sinusoidal stimuli during a short-term exposure to different concentrations of diazinon. Electrophysiological recordings from the antennules showed that neural activities in the crayfish antennules were still present 2 h after exposure to diazinon, although at decreased rates. In addition, responses to hydrodynamic stimuli indicated that the mechanosensitivity of crayfish antennules was reduced after exposure to concentrations of as low as 0.5 mg/L. The reduction in mechanosensitivity was larger and more rapid in the lateral flagellum than the medial flagellum. Observations from dye experiments showed that ambient water was more readily absorbed at the lateral flagellum than the medial flagellum probably because of differences in the setal structures of the 2 flagella. We discuss the results in relation to the possible immediate sublethal effects of diazinon on the ability of animals to search for and detect chemical and hydrodynamic cues that are important in feeding, mating, and prey/predator detection. Such information is important considering the rapid degradation of diazinon in the natural environment. <http://zoolstud.sinica.edu.tw/Journals/50.2/145.pdf>

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Disruption of crustacean behavior as a result of aquatic pollution is well documented. Most of the research conducted reported an interruption of olfactory-mediated behaviors such as foraging, predator avoidance, reproduction, and social hierarchies. For example, exposure to a blend of copper, chromium, arsenic, and selenium suppressed the preference response of the crayfish *Procambarus clarkii* and *Orconectes rusticus* to feeding stimulants (Steele et al. 1992). Similarly, exposure of *O. rusticus* to metachlor resulted not only in failure to localize a food source, but also an alteration in behavioral responses to alarm signals (Wolf and Moore 2002). Exposure to copper

affected the ability of male shore crabs *Carcinus maenas* to detect female pheromones and perform mating behaviors (Krång and Ekerholm 2006). It is notable; however, that there are few reports on the effect of aquatic pollution on another important sensory modality, i.e., the mechanosensory abilities of crustaceans. Mechanosensory setae in crustaceans detect hydrodynamic signals that carry important abiotic and biotic information such as the presence and movements of prey, predators, aggressors, and potential mates (Douglass and Wilkens 1998, Herberholz and Schmitz 1998, Hwang and Strickler 2001, Wu et al. 2010). Previous work demonstrated that

*To whom correspondence and reprint requests should be addressed. Tel: 81-99-2864242. Fax: 81-99-2864015.
E-mail: anraku@fish.kagoshima-u.ac.jp

long-term exposure to contaminants can alter the morphological characteristics of appendages and/or setae (Weis et al. 1992, Sakamoto et al. 2009), but it is yet unclear whether contaminants can influence the sensitivity of crustacean mechanoreceptors to hydrodynamic flows.

In the present study, we examined the mechanosensory properties of crayfish *P. clarkii* antennules when exposed to diazinon. Diazinon is a broad-spectrum organophosphate pesticide that is toxic to aquatic organisms and was reported to have contaminated water bodies in both developed and developing nations because of its prominent role in agriculture, veterinary medicine, and residential pest control (Larkin and Tjeerderma 2000). Like other organophosphates, diazinon is a powerful nerve poison that inhibits the activity of acetylcholinesterase (AChE), an enzyme that hydrolyzes the neurotransmitter, acetylcholine (WHO 1986). Excessive acetylcholine accumulation results in hyperactivity, abnormal swimming, spasms, growth suppression, delayed maturity, and even death in crustaceans (McKenney 1986, Roast et al. 2000, García-de la Parra et al. 2006). We examined the effects of diazinon exposure on the spontaneous neural activity of crayfish antennules. Then, we compared the thresholds of crayfish antennules to sinusoidal stimuli before and during short-term exposure to diazinon. We also investigated the morphological properties of crayfish antennules and correlated them with responses to diazinon exposure. Our results showed that diazinon reduces the sensitivity of crayfish antennules to hydrodynamic stimuli that are important sources of information on water flow and the presence and movements of prey, predators, and conspecifics. The results are discussed in relation to the ability of crayfish to detect important stimuli that influence food searching, mating, social hierarchies, and prey-predator interactions.

MATERIALS AND METHODS

Test animals

Adult American crayfish *P. clarkii*, 30-40 mm in carapace length, were obtained from a local supplier in Kagoshima Prefecture, Japan. Animals were kept in aerated tanks at 10-15°C until used for the experiments. Holding tanks were provided with *Elodea*, and animals were fed ad libitum twice a week with crayfish pellets (Japan Pet Drugs,

Tokyo, Japan).

Electrophysiological recordings

To record neural activities in crayfish antennules, a modified electrophysiological technique was used (Monteclaro et al. in press). To generate a sinusoidal dipole stimulus source, a small plastic sphere (4.5 mm in diameter) was attached to a stainless steel shaft (15 cm long and 1 mm in diameter) that was in turn connected to an oscillator (MEE15, Akashi, Kanagawa, Japan) (Fig. 1). The oscillator was driven by a function synthesizer (WF1943A, NF Electronics, Yokohama, Japan) which was controlled by a personal computer. An A/D converter (PowerLab 4/20, ADInstruments, Milford, MA, USA) was used to digitize the sinusoidal signals. The dorsal section of the distal 1/2 of the antennule was stimulated at a frequency of 5 Hz at different amplitudes which were produced by different voltages. The stimulus amplitude was measured prior to the experiment by viewing the transducer shaft movements from the side with a light microscope. The intensity of a stimulus applied to the antennule was computed in terms of the root mean square (rms) of the water particle velocity following the method of Harris and van Bergeijk (1962). The range of displacement of the sphere used in the test was 1-23 μm rms.

Crayfish were prepared for recording by immersing them in chilled water for 15 min. The antennule was cut at the base where the lateral and medial flagella bifurcated. The proximal end of the isolated antennule was inserted into a Teflon tube which was filled with van Harreveld saline solution. The isolated antennule was suspended in a 200-mL glass beaker containing previously chilled, oxygenated van Harreveld saline solution with the cut end elevated from the surface of the saline solution. The pH of the solution was 7.8, and the temperature of the saline within the recording chamber ranged 18-20°C. Nerve fibers were tested for hydrodynamic sensitivity by applying small jet streams to the flagellum or slightly touching the setae with the tip of a glass capillary, and only those fibers which showed responses were used in the actual tests.

To determine the threshold, an Ag-AgCl electrode was inserted into the Teflon tube in which the proximal end of the antennule was inserted (Fig. 1B). The pre-stimulus neural activity was recorded for 20 s, after which the stimulus was applied for at least 20 s. The electrical responses were amplified (MEG-1200, Nihon Kohden, Tokyo, Japan) and

digitized in synchrony with sinusoidal signals using the A/D converter. A speaker was used to listen to the evoked neural activities. Sinusoidal and neural data were monitored and stored in a computer (Macintosh, Apple, Cupertino, CA, USA).

Data analyses

In several cases, recordings were either dual- or multiunit recordings (Fig. 2). Discrimination of a single-unit recording was performed using the module software in Chart (Spike Histogram, ADInstruments). Data were analyzed using the same software by counting the number of spikes during the pre-stimulus and following the onset of the sinusoidal stimulus. A fiber was considered responsive to the stimulus if discharge rates obtained during stimulation were significantly higher than those during the pre-stimulus (by 2-tailed Student's *t*-test). The lowest amplitude that produced a significant increase in neural activity was determined as the threshold to water particle motion. To analyze phase locking of spikes to the sine wave, each sinusoidal wave stimulus was divided into 36° phases by adjusting the bin size to 20 ms (Fig. 2B). The number of spikes that

occurred in each phase of the sinusoidal curve was counted using the Interspike Interval Histogram (ADInstruments).

Chemical preparation and exposure

Diazinon was purchased from Wako Pure Chemical Industries (Osaka, Japan). Stock solutions were prepared with pesticide-grade acetone and stored in the dark at 4°C. Test solutions were prepared by diluting the stock solution with van Harreveld saline solution. Dose-response tests were carried out using dilutions of saline solutions containing the following diazinon concentrations: 0 (control), 0 (vehicle control), 0.5, 1.0, and 2.0 mg/L. In the vehicle control test, antennules were exposed to acetone at a concentration equivalent to the acetone concentration in the 2 mg/L diazinon solution. The antennular threshold was determined before exposure to diazinon (as a control), after which the saline solution was pumped out of the recording chamber and replaced with a previously prepared diazinon test solution using a peristaltic pump (EYELA MP-1000, Tokyo Rikakikai, Tokyo, Japan). The antennular threshold was then determined 10,

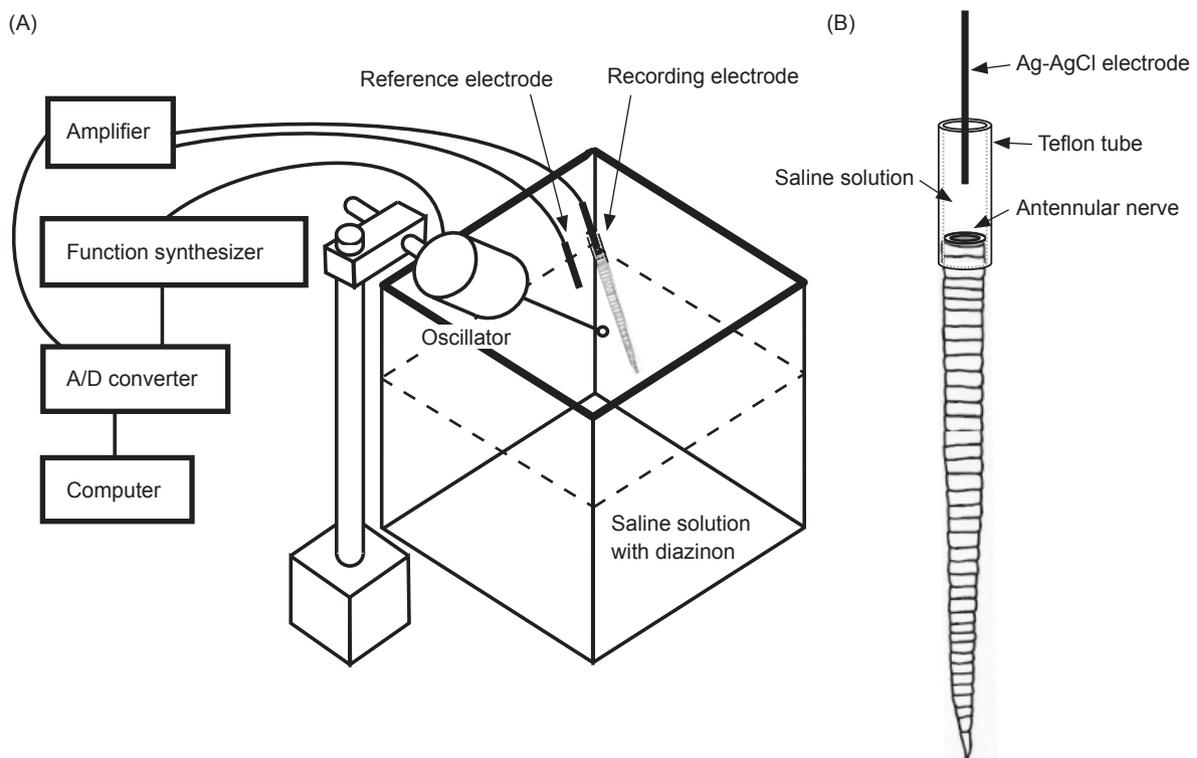


Fig. 1. (A) Experimental set-up for recording *Procamburus clarkii* antennular neural activities in response to sinusoidal stimulus before and during exposure to diazinon; (B) A closer view of the antennule and the recording electrode.

30, 60, and 120 min after initiation of exposure.

Statistical analyses

Indices used to quantify the effect of diazinon were: (1) the spontaneous neural activity and

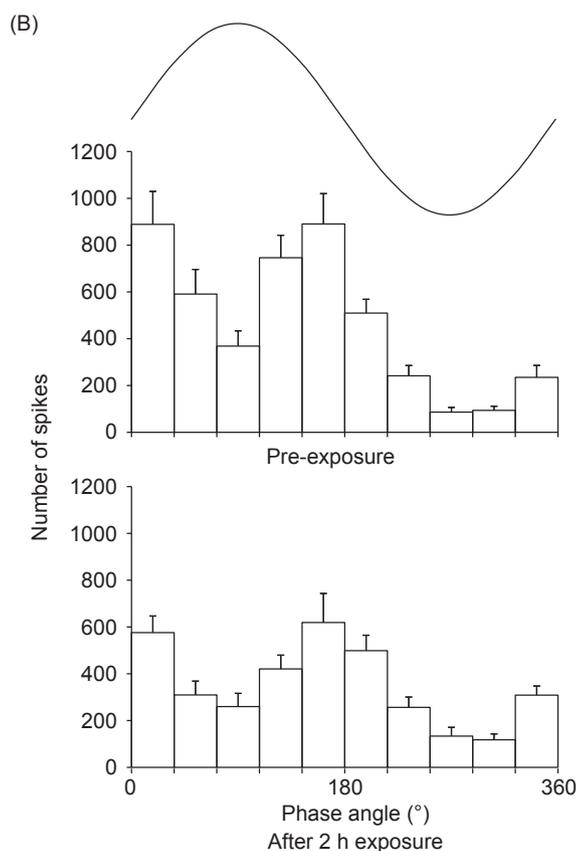
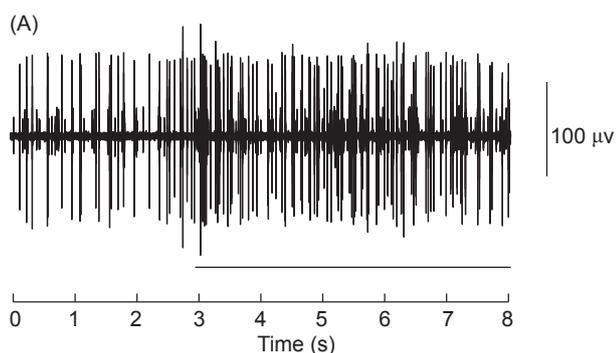


Fig. 2. (A) An example of crayfish antennular neural activity. Sensitivity of crayfish antennules to hydrodynamic stimulation is characterized by an increase in the number of neural discharges when sufficiently stimulated. Duration of stimulus is indicated by a dark line along the x-axis; (B) Phase-locking of spikes in pre-exposed (top) and exposed (bottom) antennules (bin size = 20 ms). Number of spikes indicates the total number of spikes elicited during the 20 s stimulation that occurred in specific phases along the sinusoidal wave.

(2) the threshold to sinusoidal stimuli. Student's *t*-test was used to examine whether these indices differed between the lateral and medial flagella. To test whether the different exposure periods to different diazinon concentrations significantly altered the 2 indices, a repeated-measures analysis of variance (ANOVA) was performed. Whenever there was a significant difference ($p < 0.05$), a multiple-comparison test using the Bonferroni test was used to determine significant differences between the control group (0 min exposure) and the other treatment groups ($p < 0.05$).

Porosity test

To evaluate the porosity of the cuticular structures on the antennular flagella of crayfish, a modified methylene blue staining method was used (Alexandrowicz 1951, Cate and Derby 2002). Antennules were fixed in 10% formalin in 0.2 M phosphate buffer (pH 7.4) for 24 h, rinsed in water, and exposed to 0.5% methylene blue in distilled water for 5-10 s. Then, they were rinsed in distilled water, dried under a lamp, and cleared in xylene for 15 s. To determine the absorption of dye into the flagellar setae, both the lateral and medial flagella were examined under a light microscope (Nikon Eclipse ME600, Tokyo, Japan), and photomicrographs were taken using a digital camera (Nikon E4500).

RESULTS

Spontaneous neural activity

The spontaneous neural activities recorded in the isolated crayfish antennules are shown in figure 3. Neural activities of the lateral and medial flagella were 5.06 ± 0.37 (mean \pm S.E.M.; $n = 82$) and 4.52 ± 0.35 ($n = 79$) spikes/s, respectively. The number of spikes discharged in the 2 flagella did not statistically differ ($p > 0.05$). The spontaneous neural activity rates in crayfish antennules that were not exposed to diazinon exhibited no significant changes after 2 h ($p > 0.05$). In antennules that were exposed to diazinon, neural activities were still present 2 h after exposure, although the number of discharges was reduced. Compared to the discharge rates before exposure, the mean spontaneous discharges in both flagella were significantly lower 2 h after exposure to 0.5 and 1 mg/L diazinon ($p < 0.05$).

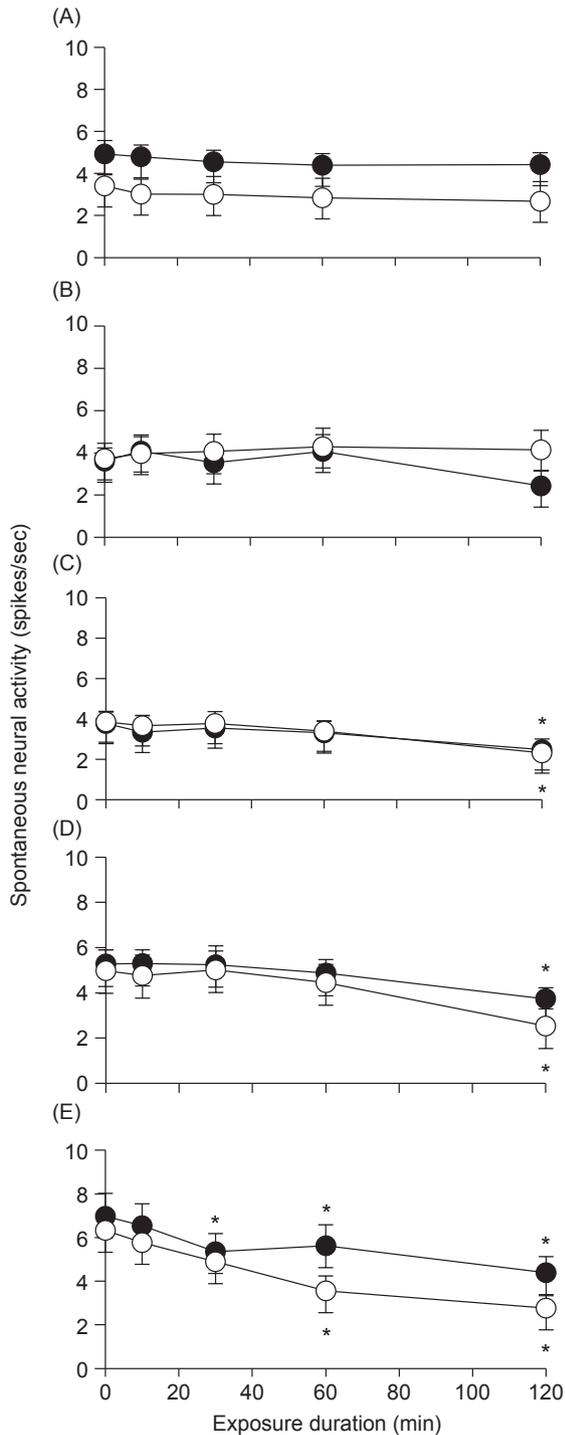


Fig. 3. The neural activities of the lateral (solid circles) and medial (open circles) flagella of crayfish antennules during diazinon exposure. Graph shows the number of spikes discharged per second during the pre-exposure period (0 min) and after 10, 30, 60 and 120 min exposure to the following diazinon concentrations: (A) 0 mg/L control; (B) 0 mg/L (with acetone); (C) 0.5 mg/L; (D) 1 mg/L; and (E) 2 mg/L. Asterisks (*) indicate significant difference from the control group following Repeated Measures ANOVA and Multiple Comparison vs. Control Group (Bonferroni test; $p < 0.05$).

With exposure to 2 mg/L diazinon, spontaneous neural activities in the lateral and medial flagella were found to have begun to decrease after 30 and 60 min, respectively.

Response to hydrodynamic stimuli

Neural activity recordings from 115 fibers showed that *P. clarkii* antennules responded to sinusoidal stimulation. When an antennule was sufficiently stimulated, there was a subsequent increase in neural discharges (Fig. 2A). All responses were tonic, characterized by a rapid increase in the discharge rate at the onset of stimulation that lasted until termination of the stimulus (Fig. 2A). The occurrence of spikes exhibited phase-locking, i.e., most of the spikes occurred at the 180° and 0/360° phases of the wave stimulus (Fig. 2B). With stimulation at 5 Hz, both crayfish antennular flagella responded to water displacement of as low as 4.29 μm rms. The normal thresholds (when not exposed to diazinon) of the lateral and medial flagella to water particle velocity ranged 59.6-248.7 and 59.6-274.7 $\mu\text{m/s}$ rms, respectively. The mean thresholds of the lateral and medial flagella (131.3 ± 5.3 (mean \pm S.E.M.; $n = 60$) and 143.5 ± 7.3 $\mu\text{m/s}$ rms ($n = 55$), respectively) did not significantly differ from each other ($p > 0.05$).

Responses during diazinon exposure

In the control and vehicle control (acetone) groups, antennular thresholds to sinusoidal stimulation before and after 2 h of exposure did not significantly differ ($p > 0.05$; Figs. 4A, B). In contrast, antennules that were exposed to diazinon exhibited an increase in the threshold to the stimuli. At 2 mg/L of diazinon exposure, the mean water velocity thresholds of the lateral and medial flagella were 168.4 ± 8.1 and 159.3 ± 7.3 $\mu\text{m/s}$ rms, respectively. This increase in the threshold suggests a reduction in the sensitivity of the antennules to hydrodynamic stimuli as influenced by the concentration of diazinon and the duration of exposure. Figure 4 shows the rate of reduction of mechanosensitivity relative to the control (pre-exposure) during short-term exposure to several diazinon concentrations. The degree of reduction in sensitivity differed between the lateral and medial flagella. The lowest diazinon concentration that caused a significant reduction in sensitivity within 2 h was 0.5 and 1 mg/L in the lateral and medial flagella, respectively. As the

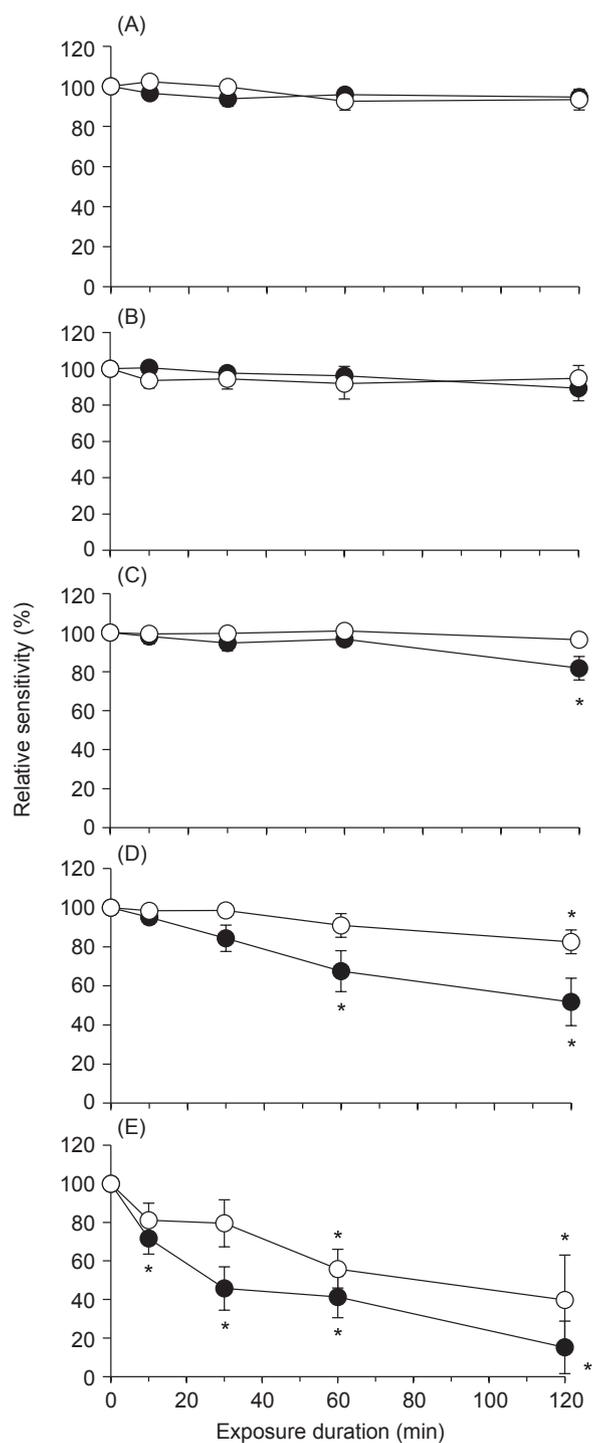


Fig. 4. Reduction in the sensitivity of the lateral (solid circles) and medial (open circles) flagella of crayfish antennules during diazinon exposure. Graph shows the relative percentage of sensitivity in comparison to the pre-exposure period (0 min) after 10, 30, 60 and 120 min exposure to the following diazinon concentrations: (A) 0 mg/L (control); (B) 0 mg/L (with acetone); (C) 0.5 mg/L; (D) 1 mg/L; and (E) 2 mg/L. Asterisks (*) indicate significant difference from the control group following Repeated Measures ANOVA and Multiple Comparison vs. Control Group (Bonferroni test; $p < 0.05$).

diazinon concentration increased, the reduction in sensitivity became more rapid. At 1 and 2 mg/L of exposure, reductions in the lateral flagellum sensitivity were observed after 60 and 10 min, respectively. In contrast, exposure of the medial flagellum to 2 mg/L diazinon caused a reduction in its sensitivity after 1 h. Diazinon-exposed antennules retained their tonic and phase-locking properties in response to sinusoidal stimuli (Fig. 2B). No data were available for exposure for more than 2 h because the viability of the isolated antennules was greatly reduced, i.e., spontaneous discharge activities began to weaken 2 h after the antennule had been cut and therefore was deemed unsuitable for further recording use.

Porosity test of crayfish antennular setae

Crayfish antennules are biramous, with 1 lateral and 1 medial flagellum (Fig. 5A). Both flagella contained a number of setae although the chemoreceptive aesthetascs and their associated setae were present only on the ventral surface of the lateral flagellum's distal 1/2 (Fig. 5B). Non-aesthetasc setae included procumbent and standing plumose setae, smooth conate setae, and short, medium, and long simple setae. The procumbent plumose setae, standing plumose setae, and smooth conate setae were usually present on the proximal 1/2 of both the lateral and medial flagella. The medium and long simple setae were distributed along the dorsal surface of the lateral flagellum (Fig. 5B) and on the distal 1/2 of the medial flagellum (Fig. 5C). Among the different setal types, the procumbent plumose setae were the largest and most numerous (Fig. 5D).

Immersion of crayfish antennules in methylene blue showed that the different setae on the antennular surface had different porosity properties. Within 5-10 s of exposure to methylene blue, the distal end of the aesthetascs were darkly stained (Fig. 5F). In contrast, procumbent (Fig. 5E) and standing plumose setae tested negative for the methylene blue dye. Similarly, the medium and long simple setae were not stained (Figs. 5G, H). These results imply that the aesthetascs are more permeable and suggest a faster rate of absorbing chemicals that are present in the ambient water.

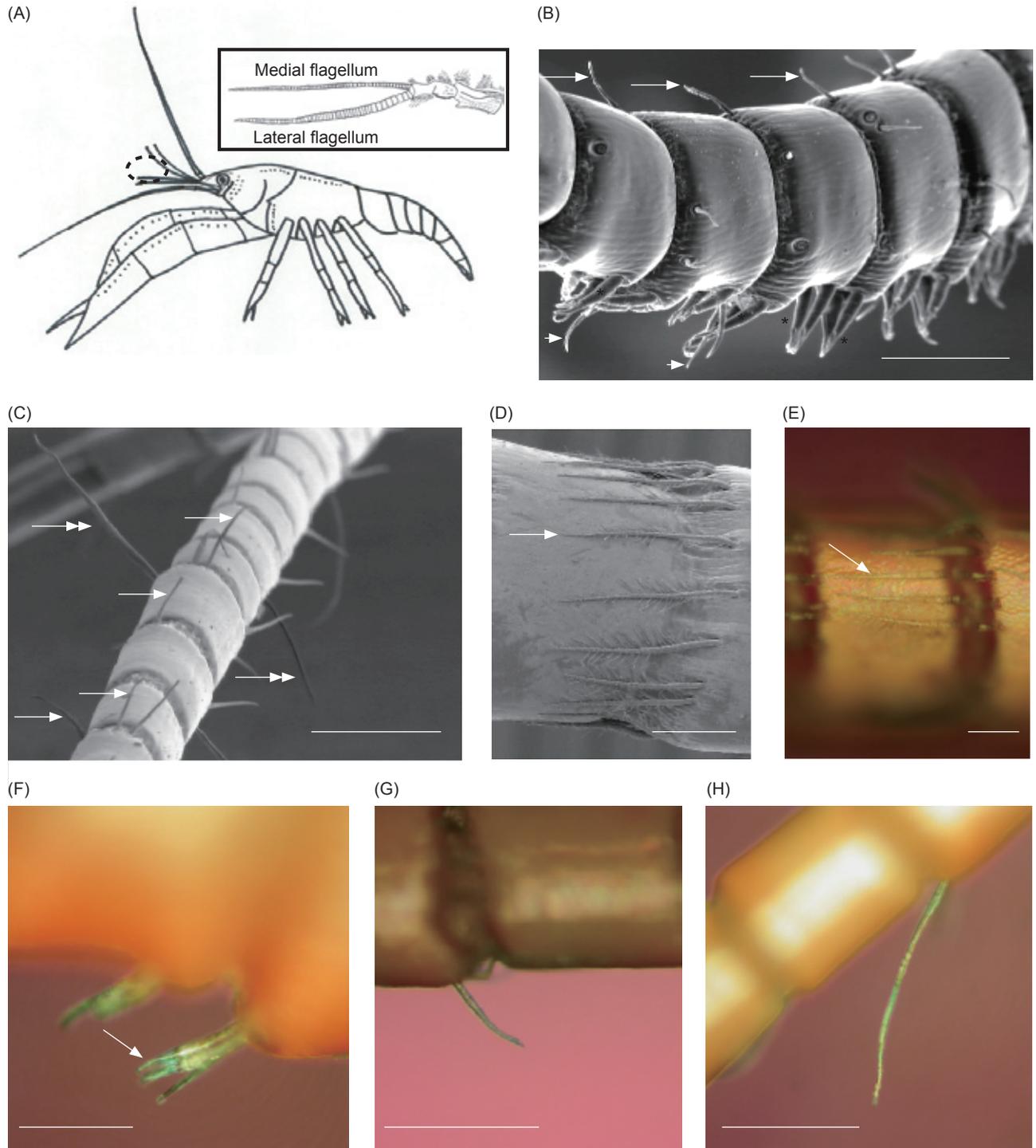


Fig. 5. (A) Illustration of a crayfish showing the biramous antennules with lateral and medial flagella; (B) The distal half of the lateral flagellum with chemoreceptive aesthetascs (asterisks) and associated setae (arrowhead) present on the ventral surface, and mechanoreceptive medium simple setae (arrow with single head) on the dorsal surface; (C) The distal half of the medial flagellum with long (arrow with double heads) and medium (arrow with single head) simple setae; (D) The procumbent plumose setae (arrow); (E) Stained procumbent plumose setae (arrow); (F) Dye stains on the distal ends (arrow) of aesthetascs; (G) Medium simple setae negative to staining; (H) Long simple setae negative to staining. Scale bars: B-D = 150 μm; E-H = 200 μm.

DISCUSSION

The results of this study demonstrate that diazinon reduces the mechanosensitivity of crayfish antennules to fluid flow. Owing to the relatively large size and cuticular walls of crayfish mechanosensory setae, diazinon seems most likely to exert its actions via the afferent system and not, for instance, via transduction channels. This can be confirmed by the preservation of the tonic and phase-locking properties of the setae to stimuli. Also, other physical properties affecting setal shaft motion, like the fluid viscosity and fluid density, are very unlikely to have been changed by the diazinon concentrations used. Furthermore, the setal number, shape, and volume remained unchanged after exposure. Diazinon must have disturbed the afferent system as suggested by the differences in post-exposure threshold rates between the lateral and medial flagella. The lateral flagellum, which can absorb ambient water through the numerous chemoreceptors along its surface, exhibited a faster and larger increase in the threshold compared to the medial flagellum. It is unclear whether diazinon undergoes axonal transport in crayfish, but in fish and rats, the transport of waterborne contaminants from the olfactory epithelia towards the next-order neurons and ultimately the brain was reported (Sloman and Wilson 2006). Diazinon is a known inhibitor of acetylcholine hydrolysis (WHO 1986). In the crayfish neural system, acetylcholine was reported to be a neurotransmitter in synapses between mechanoreceptors in the exopodite and interneurons in terminal ganglia (Ushizawa et al. 1996) and in synapses between abdominal mechanoreceptors and their 1st-order interneurons in the crayfish lateral giant neuron escape system (Miller et al. 1992). Although acetylcholine is abundant in lobster antennules (Barker et al. 1972, Hildebrand et al. 1974), the actual neurotransmitters involved in synapses between crayfish antennular mechanoreceptors and next-order interneurons have yet to be identified. Nevertheless, our results showed that exposure of the antennules to diazinon resulted in a reduction in spontaneous neural activities, as opposed to the continuous firing of nerve impulses when acetylcholine accumulates in nerve tissues after AChE inhibition (Gaworecki et al. 2009). This suggests that at this level of peripheral sensory transmission in antennules, diazinon exerted its action on electrical transmission.

Both the lateral and medial flagella were

sensitive to hydrodynamic stimuli with threshold values comparably similar to values reported by Monteclaro et al. (2010). Monteclaro et al. (2010) showed that spikes occurred close to the 0°/360° phase of the sinusoidal wave, with the degree of phase-locking lowest at 10 Hz and highest at 200 Hz. It seems that at much-lower frequencies, spikes also occurred at the 180° phase of the wave, possibly because of the influence of the velocity component of the water stimulus on hair receptor properties such as the shaft length and width, and degree of deflection. The sensitivity of both antennular flagella to hydrodynamic stimuli suggests their capability to detect animal-generated water movements, the main spectral amplitudes of which are mostly < 10 Hz (Kalmijn 1989, Bleckmann et al. 1991). It was shown that mechanoreceptors can perceive the swimming movements of fish as demonstrated in the response of blindfolded *P. clarkii* (Breithaupt et al. 1995). In addition, hydrodynamic stimuli may also provide crayfish with information on self-generated flows (Breithaupt 2001) or water flows generated by conspecifics that are linked to mating and agonistic behaviors (Breithaupt and Eger 2002, Bergman et al. 2005). Furthermore, hydrodynamic stimuli provide crayfish with the initial stimulation to commence antennular flicking (Monteclaro et al. 2010), a decapod crustacean behavior that is believed to enhance detection of chemical cues by the aesthetascs (Schmitt and Ache 1979). When diazinon disrupts the sensitivity of crayfish antennules to hydrodynamic signals, all of these mechanosensory-related functions may be compromised.

The rapid absorption of dye into the aesthetascs suggests that the lateral flagellum exhibits greater receptivity to diazinon compared to the medial flagellum. This poses a potential threat to the functions of the aesthetasc, which is acknowledged as a chemoreceptor that influences crustacean behavior in food searching (Laverack 1988), mating (Tierney et al. 1984, Kamio et al. 2005), prey-predator interactions, and agonistic and social behaviors (Horner et al. 2008). Chemosensory deprivation in crayfish could adversely affect feeding, growth, and reproduction. Diazinon was reported to reduce the ability of the shrimp (*Metapenaeus ensis*) to locate food sources (Chu and Lau 1994). In fish, this pesticide was reported to decrease the ability of hybrid striped bass (*Morone saxatilis* × *M. chrysops*) to capture prey (Gaworecki et al. 2009), and disrupt antipredator and homing behaviors in Chinook

salmon (*Onchorhynchus tshawytscha*; Scholz et al. 2000) and sexual odor recognition in mature male Atlantic salmon (*Salmo salar*; Moore and Waring 1996).

Our study shows that exposure to diazinon resulted in a reduction of crayfish antennular sensitivity to hydrodynamic signals at levels that are lower than acute values reported in crayfish. Morgan (1976) reported that the 24 h LC₅₀ of diazinon to crayfish *O. propinquus* was 2846 µg/L, with 2332 and 3360 µg/L as the respective minimum and maximum toxicity values. The effect of diazinon on crayfish mechanoreception and probably on olfaction as well was swift, especially on the lateral flagellum. This information is particularly important because diazinon in the natural environment easily degrades (Larkin and Tjeerderma 2000), thus immediate effects on the physiology of affected organisms need to be studied. Based on our results, it seems that diazinon concentrations of < 0.5 mg/L have the potential to reduce the sensitivity of crayfish antennules to hydrodynamic stimuli, albeit gradually. We did not determine the time course of recovery of crayfish antennules to diazinon exposure because of experimental design constraints. For crayfish, the relative rates of recovery for biochemical and behavioral endpoints have not been investigated. However, data on longer-term physiological and behavioral impairment after acute exposure to diazinon are available. For example, evidence from fish suggests that the activities of AChE and other cholinesterase's in tissues may be inhibited for days to weeks, even if animals no longer contain detectable traces of pesticides (Cong et al. 2008). This may be an important ecological consideration given the seasonal and intermittent transport of organophosphate pesticides. Also, the use of organophosphate pesticides is increasing as a result of restrictions on organochlorines, which are very persistent in the environment and have a tendency to significantly bioaccumulate through food chains (WHO 1986).

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