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Title: Cardiovascular and Respiratory Responses of the Ghost Shrimp, Callianassa californiensis Dana, to the Pesticide Carbaryl and its Hydrolytic Product 1-Naphthol

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Many environmental factors influence cardiovascular and respiratory activities of crustaceans. The effects of natural stressors (hypoxia, low salinity, high temperature) have been well studied, but the effects of pollutants upon these two organ systems have received less attention. The presence of the pesticide carbaryl (Sevin, Union Carbide, Inc.) and its degradation product, 1-naphthol, in Northwestern rivers and bays has resulted from intentional application to commercial oyster beds and through careless handling during other spraying operations. In this study, the burrowing thalassinid ghost shrimp, Callianassa californiensis was exposed to sublethal levels of the two toxicants to assess their influence on the cardiovascular and respiratory performance of these tidal mudflat dwellers.

Both carbaryl and 1-naphthol altered the behavior of exposed ghost shrimp, causing hyperactivity, convulsions and paralysis. During these changes oxygen consumption was increased two-fold. In carbaryl-treated ghost shrimp, normally occurring periods of apnea

were abolished and replaced with high frequency scaphognathite beating. The heart rate, however, was not substantially increased. Once carbaryl treatment was discontinued, scaphognathite rates declined and heart rates became elevated. During 1-naphthol exposure, neither heart nor scaphognathite activities were consistently altered. Both toxicants stimulated the secretion of mucosubstances by the shrimp, however, the source of these secretions is not known.

The responses of ghost shrimp to carbaryl and 1-naphthol may be due, in part, to the ability of these compounds to reversibly inhibit acetylcholinesterase, the enzyme which degrades acetylcholine at cholinergic nerve synapses. Although the exact mode of action of these two toxicants in ghost shrimp is not known, it is clear that any substance which would alter their behavior and increase their metabolic demands might seriously affect the ability of the ghost shrimp to survive in the hypoxic mudflat environment.

Cardiovascular and Respiratory Responses of the Ghost Shrimp, *Callianassa californiensis* Dana, to the Pesticide Carbaryl and its Hydrolytic Product 1-Naphthol

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GENERAL INTRODUCTION

Insecticides, both through intentional application and through careless handling, have become common contaminants of estuarine systems. More than 20 years ago the carbamate insecticide carbaryl (1-naphthyl methylcarbamate; Sevin, Union Carbide, Inc.) was approved for use on tidal mudflats in commercial oyster beds in Washington State to eradicate ghost shrimp, *Callianassa californiensis*, where their burrowing activities tend to create improper substrate for young oysters (Lindsay, 1961). Although carbaryl has not been approved for use in oyster beds in Oregon, it has been applied extensively to forests to control spruce budworm and gypsy moth infestations. Accidental spills associated with these spraying operations resulted in the contamination of rivers in Oregon (The Oregonian, 19 June 1983; Corvallis (OR) Gazette-Times, 14 July 1983). Run-off from forests and agricultural fields may also increase the presence of carbaryl in Northwestern rivers and bays.

The thalassinid ghost shrimp, *Callianassa californiensis* is one of the most abundant species inhabiting the mixed sand and mud sediments of coastal bays from southern Alaska to southern California (MacGinitie, 1934). McCrow (1971) estimated the density of ghost shrimp to be 3-6 million per acre in Yaquina Bay, Oregon. While

constantly burrowing in the sediment to feed on detritus the ghost shrimp aerate the sediment and keep the top layers well mixed. This creates the proper habitat for many other mud flat species (MacGinitie, 1934). Except for use as fish bait, ghost shrimp have no commercial value, however their role as the "earthworms" of the mudflat appears to be essential in maintaining the mud flat community. When mudflats are treated with carbaryl, recolonization by ghost shrimp may not occur for up to 18 months (Lamberton and Claeys, 1970) and the reestablishment of the community may take considerably longer.

Carbaryl is relatively unstable under the conditions found in the estuarine environment (Karenin et al., 1967; Aly and Ei-Dib, 1971). In alkaline seawater carbaryl is hydrolyzed to 1-naphthol. In a field study conducted by Karenin et al. (1967) carbaryl was applied to an intertidal mudflat. Two hours after application carbaryl and 1-naphthol were found in equal concentrations in the top layer of sediment. Kanazawa et al. (1975) showed that although aqueous carbaryl is unstable, carbaryl may become concentrated in the soil where physical decomposition becomes slower. Therefore, ghost shrimp and other mud dwellers may be subjected to higher concentrations of carbaryl than are found in the water column. 1-Naphthol also degrades at alkaline pH, in addition, photodecomposition may occur in aqueous solutions exposed to sunlight (Lamberton and Claeys, 1970). Soil microorganisms are able to transform carbaryl into 1-naphthol and other products (Mount and Oehme, 1981), and are also able to metabolize 1-naphthol under

aerobic conditions (Liu et al., 1981; Lamberton and Claeys, 1970). There is evidence that one of the products of 1-naphthol degradation is persistent in the environment and is toxic to marine organisms though its exact chemical composition is not known (Lamberton and Claeys, 1970).

Stewart et al. (1967) found that crustaceans were more sensitive to carbaryl than were fish or molluscs and that ghost shrimp were the most sensitive of the crustaceans tested to 1-naphthol. 1-Naphthol, however, was not as toxic to crustaceans as it was to molluscs and fish. Insecticides have been developed with toxicity to insects in mind, but since crustaceans are closely related to insects they would probably be physiologically more susceptible to insecticides than would fish or molluscs. The reason why fish and molluscs are more sensitive to 1-naphthol is not known (Stewart et al., 1967).

The most widely accepted theory of carbamate insecticide toxicity is based upon their ability to inhibit acetylcholinesterase (AchE), the enzyme which degrades acetylcholine (Ach) at cholinergic nerve synapses (Kuhr and Dorough, 1976). Figure 1 outlines the interaction of carbaryl with AchE. Carbaryl enters the synapse and competes with Ach for the active site on the enzyme. Once covalent binding of carbaryl to a serine residue in the active site occurs (Forsberg and Puu, 1984), an intermediate complex is formed (step +1). This intermediate complex may dissociate back to the enzyme and intact substrate (step -1) or the enzyme may be carbamylated, releasing a 1-naphthol leaving group (step 2). Hydrolysis

(decarbamylation) leads to free enzyme plus methyl carbamic acid (step 3). Carbaryl inhibits AchE because the rates of carbamylation (step 2) and hydrolysis (step 3), especially the latter, are much slower when carbaryl is the substrate compared with Ach. Carbaryl, therefore, competitively inhibits the enzyme (half life for hydrolysis is 30-40 min), preventing it from interacting with Ach at the synapse. Accumulation of Ach at the synapse can lead to prolonged stimulation of postsynaptic nerve or muscle cells and lead to disturbances in nerve and muscle function. The mode of action of 1-naphthol has not been widely studied and so the reason for its toxicity is not understood, but it does have some ability to inhibit AchE (Kuhr and Dorough, 1976).

The purpose of my investigation is to describe the effects of sublethal levels of carbaryl and 1-naphthol in ghost shrimp. Although ghost shrimp are occasionally exposed to high levels of these toxicants, it is likely that low levels would be encountered more often. Even sublethal levels may cause serious disturbances in the physiological functioning of the animals, leaving them unable to cope with the demands of their environment. I chose to monitor the activities of the cardiovascular and respiratory systems, in particular, for the following reasons:

1. The cardiovascular and respiratory systems are very sensitive to changes in the overall physiological status of crustaceans. This is apparent from studies examining the effects of other pollutants as well as stressful environmental conditions such as low salinity, hypoxia, and high temperature.

2. The activities of the heart and scaphognathites (gill-bailers) in crustaceans are regulated at several levels by the nervous system (Wilkens, 1981). The role of cholinergic nerves in controlling rhythmic behaviors such as these is not well understood (Wiens, 1982; Atwood, 1982). Changes in heart and scaphognathite function resulting from treatment with carbaryl could contribute to our understanding of the role of Ach as a neurotransmitter in crustaceans.

3. Many neurotoxic insecticides change the energetic requirements of animals by affecting muscle activity and/or behavior. In addition, they may modify many metabolic processes by altering circulating hormone levels (Atwood, 1982; Singh and Orchard, 1982; McMahon and Wilkens, 1983). Alterations in metabolic rate and energy utilization can be detected by measuring oxygen consumption rates.

4. Many pollutants first interact with aquatic animals at the surface of the gills because the gill epithelium is in direct contact with the external environment and is usually relatively unprotected. Alterations in the properties of the gill epithelium as a result of exposure to a toxicant could lead to changes in ventilation pattern or oxygen uptake (Thurberg, 1980).

In this study the relative toxicities of carbaryl and 1-naphthol to ghost shrimp under laboratory conditions were assessed and the behavioral changes caused by the two toxicants were described (Chapter 1). Then the normal patterns of heart and scaphognathite activity were examined prior to the investigation of the changes caused by carbaryl and 1-naphthol treatment (Chapter 2). The release

of mucosubstances by intoxicated ghost shrimp was quantified (Chapter 3), and finally, oxygen consumption rates in treated ghost shrimp were determined (Chapter 4).

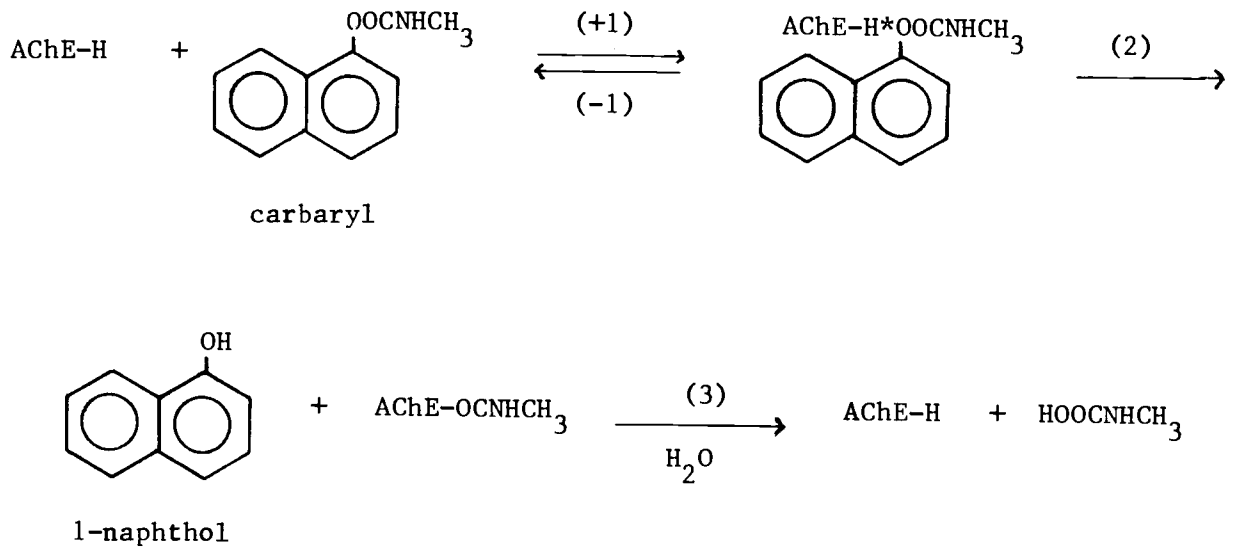

Figure 1. Interaction of carbaryl with acetylcholinesterase (AchE-H; from Kuhr and Dorough, 1976). The numbers in parentheses allow each reaction to be referred to by number in the text.

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CHAPTER 1

The toxicity of carbaryl and 1-naphthol to the ghost shrimp, *Callianassa californiensis*

INTRODUCTION

When the toxicity of a pollutant to a particular species is unknown, it is necessary to conduct bioassays prior to further investigations. The toxicant concentration proving lethal to 50% of the test organisms (LC_{50}) is most often used to report the sensitivity of aquatic organisms to toxicants. When death is difficult to judge or when other responses are deemed more appropriate, the LC_{50} is often replaced by the EC_{50}, the effective toxicant concentration producing a particular response in 50% of the test organisms. Closely related groups of animals, even those of the same species, may display a range of sensitivities to toxicants (American Public Health Assoc., 1980). Previously reported toxicity data may be unreliable, especially in cases where experimental conditions and life stages vary.

Short-term bioassays are often used to determine the toxicity of substances which may be released in pulses and flushed from the system, or with substances which degrade rapidly in the environment, as is the case with carbaryl and 1-naphthol in an estuarine system. Although flow-through bioassays are usually preferable to static bioassays because waste products are removed and toxicant

concentrations are maintained, a static system was chosen in this study due to a limited seawater supply. When the biomass per container is minimized and the metabolic rate of the test organism is low as it is in ghost shrimp (Thompson and Pritchard, 1969), a short-term static bioassay can be justified (American Public Health Assoc., 1980).

The purpose of the bioassays was to determine the toxicity of carbaryl and 1-naphthol to ghost shrimp over an interval corresponding to the period of highest concentration in the environment following application (Karenin et al., 1967). Behavioral changes associated with carbaryl and 1-naphthol intoxication and the time course of their development is described in this study. These behavioral changes are used subsequently to aid in the elucidation of the cardiovascular and respiratory effects of the two toxicants in ghost shrimp.

MATERIALS AND METHODS

Ghost shrimp, Callianassa californiensis, were collected from the tidal mud flats at Alsea Bay, Waldport, Oregon using a manual suction device called a "shrimp gun". The ghost shrimp were immediately transported to a recirculating seawater system and held at 14 ± 2°C and 30 ± 2 ppt salinity in a tank containing sediment acquired from the mud flats. The sediment allowed the ghost shrimp to burrow and feed while held in the laboratory. Both males and nongravid females were used in the assays since Stewart et al. (1967)

found that males and females were equally sensitive to carbaryl and 1-naphthol. Sex ratios used in the bioassays were similar to those in the population collected and sizes ranged from 0.7 to 17.2 g ($\bar{x}$ = 6.3 g). Only animals displaying normal behavior and apparently free from disease and parasites were selected for the assays.

Shrimp in equal groups of 4 to 7 individuals (depending upon availability) were placed in 14 l of seawater in 5 gal aquaria without sediment. Seawater was obtained from the recirculating system and was kept well aerated at 12.2 ± 0.1°C in an environmental chamber. The average salinity was 30.7 ± 0.2 ppt and the average pH 7.89 ± 0.02 These conditions closely resemble the average temperature, salinity, and pH of the ghost shrimps' habitat (McCrow, 1971). Although the use of sediment in the tanks would have created a more realistic environment it would have complicated the toxicity determinations due to adsorption of the toxicants onto the sediment particles, thereby decreasing concentrations in the water over time (Kanazawa et al., 1975). Shrimp in each group were matched by size and sex so that each tank contained similar biomass and sex ratios. Shrimp were allowed to acclimate to the test conditions for 24 hr prior to the introduction of toxicant. Constant illumination was maintained to minimize photoperiod effects.

Carbaryl, as Sevin 80S, was supplied by Union Carbide Agricultural Products Co, Inc. (Research Triangle Park, NC). This formulation consisted of a wettable powder containing 80% active and 20% inert ingredients. Analytical grade (99.4%) Sevin was used in determining standard curves and was also supplied by Union Carbide.

Grade III (99+%) 1-naphthol was used both in the bioassays and as a standard and was obtained from Sigma Chemical Co. (St. Louis, MO).

Toxicant stock solutions were prepared by adding the toxicants to seawater and stirring vigorously for approximately 1 hr at 4°C. Both carbaryl and 1-naphthol are only slightly soluble in water and tend to degrade at high temperatures. The solutions were then filtered (0.45 μ) to remove any undissolved material. The actual concentration of the stock was determined colorimetrically using the method of Miskus et al. (1959) and Asperen (1962) as modified by Karinen et al. (1967). This method makes use of the interaction of diazonium salts with aromatic compounds containing electron-releasing groups to form azo compounds. The diazoblue dye (0-dianisidine, tetrozotized, zinc chloride complex) was obtained from Sigma Chemical Co. and the absorbance of the samples was read at 600 nm in a 1 cm cell, using a Varian Techtron UV-Vis spectrophotometer (Model 635; Walnut Creek, CA). In carbaryl stock solutions, the initial 1-naphthol content was always below the level of detection so that the carbaryl content could be determined directly. Standard curves were prepared using serial seawater dilutions of a known quantity of either analytical grade Sevin or Grade III 1-naphthol dissolved in ethanol. The carbaryl and 1-naphthol standard curves were linear over the ranges of 0-49 mg/l (243 μmole/l) and 0-35 mg/l (243 μmole/l), respectively (see Appendix, Figure 18).

Once the concentration of a stock solution was determined, the appropriate volume of stock was added to each aquarium to yield the proper test concentration. Thorough mixing was accomplished by the

aerators in the tanks. Samples were removed for determination of the actual initial concentration in each tank. Carbaryl samples had to be concentrated before analysis due to the limited sensitivity of the assay. Concentration was accomplished by passing 100-500 ml of test medium through a Baker-10 SPE disposable octadecyl extraction column (reverse-phase octadecylsilane bonded silica gel; J.T. Baker Chemical Co., Phillipsburg, NJ). The carbaryl bound to the column was eluted with methanol and was analyzed as previously described. The standard curves using methanol rather than seawater as a solvent were identical to the seawater standard curves. Concentrations of the toxicants were again determined at the conclusion of the assay.

Shrimp were exposed to the toxicants for 24 hour. During the course of some of the bioassays the behavior of each ghost shrimp was noted at regular intervals, so that the stages of intoxication could be described. The loss of equilibrium or normal upright posture was chosen as an easily distinguishable behavioral end point for the assays since the degree of paralysis could not be determined reproducibly, and death was not readily distinguishable from complete paralysis. The EC_{50} and EC_{100} values (the effective concentration resulting in loss of equilibrium in 50% and 100% of the animals) were determined by fitting a sigmoid curve to the plotted data. For comparison, the data was subjected additionally to probit analysis.

RESULTS

Carbaryl- and 1-naphthol-exposed ghost shrimp exhibited similar behavioral changes during the course of intoxication. The different stages of intoxication are described in Table 1. During the bioassay ghost shrimp were judged on their ability to maintain equilibrium or normal upright posture. In Stages 1-2, ghost shrimp were able to maintain a normal stance and were unable to do so in Stages 3-5.

The results of the bioassays are summarized in Figure 2. The EC_{50} of carbaryl was 0.0825 mg/l (Fig. 2a) and that of 1-naphthol was 3.52 mg/l (Fig. 2b), as determined from best fit sigmoid dose-response curves. Probit analysis yielded similar EC_{50} values of 0.0814 mg/l for carbaryl and 3.62 mg/l for 1-naphthol. The asymptotic EC_{100} estimated for carbaryl was 0.150 mg/l and for 1-naphthol was 7.80 mg/l. The EC_{100} ($EC_{99.99}$ since the scale never reaches 100) values obtained through probit analysis were 0.175 mg/l for carbaryl and 15.35 mg/l for 1-naphthol. Though this procedure yields a slightly higher EC_{100} for carbaryl and a much higher EC_{100} for 1-naphthol, the values obtained from the sigmoid curve correspond to the $EC_{98.4}$ for carbaryl and the $EC_{92.4}$ for 1-naphthol on the probit scale. The discrepancy is due to the asymptotic nature of the dose-response curve.

When exposed to the 24 hr EC_{50} of carbaryl or 1-naphthol, ghost shrimp typically displayed Stage 2 or 3 behavior by the end of the dose period. Few individuals reached Stage 4 at this concentration. At the EC_{100} level, carbaryl and 1-naphthol

exposed ghost shrimp usually displayed Stage 2 behavior by 3-6 hr, Stage 3 behavior by 6-9 hr and some individuals displayed Stage 4 by 18-21 hr. Only ghost shrimp exposed to carbaryl concentrations exceeding 1.0 mg/l or 1-naphthol concentrations exceeding 20 mg/l ever exhibited Stage 5 behavior. Death could not be positively determined for any ghost shrimp at any concentration of either toxicant during 24 hr of exposure. None of the control ghost shrimp exhibited any signs characteristic of intoxication, nor did any mortality of control animals occur during the acclimation or test periods.

There is considerable variability in the response of ghost shrimp to toxicant concentrations near the middle of the range tested (see Fig. 2). This variability does not appear to be sex- or size-specific in the case of carbaryl, but may be for 1-naphthol. Figure 3 shows size (weight) profiles for the ghost shrimp treated with carbaryl and 1-naphthol grouped according to sex and their response to the range of concentrations producing greater than 0% loss of equilibrium, but less than 100% loss of equilibrium. In other words these graphs display which ghost shrimp, according to size and sex, maintained or lost equilibrium, in groups in which some individuals succumbed to the toxicant but others did not. As a group both carbaryl- and 1-naphthol-exposed male ghost shrimp were larger than the female ghost shrimp ($p<0.01$). However, there was no difference in the mean weights of ghost shrimp which failed to maintain equilibrium when compared with those which maintained equilibrium during exposure to either toxicant. When male and female

components of the carbaryl-exposed group were examined separately (Figure 3a), approximately equal numbers of males and females lost equilibrium as maintained it, and the mean weight of the ghost shrimp in the two behavioral categories were equal as well. On the other hand, only 36% of the females exposed to 1-naphthol lost equilibrium as compared with 49% of the males (Figure 3b). These males, however, were larger than the males which were able to maintain upright posture ($p<0.05$). In summary, no relationship could be discerned between gender, weight, and response to carbaryl. There appears, however, to be a slightly greater sensitivity of the larger males to 1-naphthol, with smaller males and a majority of females (64%) showing a greater tendency to maintain normal posture during 1-naphthol exposure.

Tissue samples were not analyzed to determine the amount of toxicant taken up by the ghost shrimp, nor was any attempt made to identify metabolites of either compound. There was a 17% loss of toxicant in the covered tanks over the 24 hr period. This loss may be attributed to uptake by the ghost shrimp themselves since virtually no loss was noted in tanks which did not contain ghost shrimp, whether they were aerated or not. Loss of toxicant from the medium mimics the situation in the environment. Thus when toxicity tests are performed, the sensitivity of the organisms may be overestimated if the toxicant level is held constant artificially (Stanley and Trial, 1980). Aquaria containing carbaryl did not show appreciable levels of 1-naphthol at the end of 24 hr.

DISCUSSION

Using irreversible paralysis as the criterion, Stewart et al. (1967) obtained 24 hr EC_{50} values in ghost shrimp of 0.13 and 6.6 mg/l for carbaryl and 1-naphthol, respectively. They tested both males and females ($\bar{x}$ = 2.6 g) at 20° C, in seawater of 25 ppt salinity and pH 8.0. These EC_{50} values correspond more closely to the EC_{100} values obtained in this study. The discrepancy is probably caused by the difference in the behavioral response judged. Differences in the temperature and the size of the test animals used in the two studies may have somewhat influenced the values obtained.

Twenty-four hr LC_{50}, TL_m (median tolerance limit, largely equivalent to the LC_{50}) and EC_{50} (paralysis) values have been determined for carbaryl in a wide variety of marine and freshwater arthropods. Some of these are listed in Table 2. There is great variance in sensitivity which may be due, in part, to variations in experimental conditions or size of the test animals. However, wide variances have also been reported under controlled conditions for insects that are of similar size. These differences in sensitivity have been attributed to differences in detoxification mechanisms naturally present in insects (Mount and Oehme, 1980). For example, the LD_{50} (topical dose) for houseflies can be as high as 900 µg/g while that for honeybees is 2.3 µg/g (Metcalf et al., 1967). The toxicity of 1-naphthol has not been as widely studied. Only Stewart et al. (1967) have reported EC_{50} values for aquatic crustaceans other than ghost shrimp. They report mean values of 77.2

and 47.8 mg/l for shore crabs (Hemigrapsus oregonensis) and Dungeness crabs (Cancer magister), respectively.

Behavioral changes observed in insects exposed to carbaryl have been attributed to AchE inhibition. Cholinergic synapses are common throughout the insect CNS, but seem to be particularly numerous in the thoracic ganglion (Kuhr and Dorough, 1976). The actual role of Ach in controlling various activities has been hard to elucidate because of the insensitivity of the CNS to applied Ach (Pichon, 1974). The neuromuscular junctions of insects do not seem to be cholinergic since nicotine does not produce tetanic responses in isolated muscle as it does in nerve-muscle preparations (Pichon, 1974).

Many insecticides may alter the behavior of insects by interfering with cholinergic synaptic transmission. DDT, for example, stimulates the release of Ach from sensory neurons and induces muscle hyperactivity through reflex arcs (Keister and Buck, 1974). Organophosphates (OPs) are irreversible inhibitors of AchE and may also cause hyperactivity, convulsions, and paralysis (Kuhr and Dorough, 1976). At least two aspects of carbamate poisoning may distinguish it from poisoning by other insecticides. Carbamates are comparatively fast acting and carbamate-induced paralysis is quite often reversible (Miller ,1976). Death as a direct result of carbamate poisoning is then hard to explain.

Alternative modes of action have been explored to explain insecticide-induced behavioral changes and death in insects. Early investigators noted that insects subjected to various forms of

physical stress often became paralyzed and displayed other behavioral changes similar to intoxicated insects (Heslop and Ray, 1959). Now it is believed that the release of neurohormones from neurosecretory cells is responsible for some of the signs seen in the insect stress response and in insecticide intoxication (Maddrell and Reynolds, 1972). The neurohormone release may be a consequence of nervous system hyperactivity (Orchard and Osborne, 1979). Other studies suggest that the neurosecretory cells are more sensitive to insecticides than other nerve cells since release of neurohormones prior to the development of nervous system hyperactivity has been documented (Singh and Orchard, 1982). Death may result from hormonal perturbations rather than nervous system impairment.

Ach has long been suspected to be a neurotransmitter of the CNS in crustaceans, but cholinergic synapses have been hard to identify. Ach has been found in the ganglia, in certain nerve fibers and in the eyestalks (Atwood, 1982), and AchE is well distributed throughout the nervous system especially in glial sheaths and in the neuropil areas of the central ganglia (Maynard, 1971). However, Ach only completely fulfills the criteria of a neurotransmitter in sensory nerves and in some stomatogastric motor neurons (Atwood, 1982; Wiens, 1982).

Although the mechanism is not clear, the hyperactivity, convulsions, loss of equilibrium, and paralysis seen in the ghost shrimp in this study, in freshwater shrimp (Naqui and Ferguson, 1970), and in Dungeness crabs (Armstrong, 1974) could be caused by the inhibition of AchE by carbaryl. Flory (1973) collected measurable quantities (2×10^{-9} g/ml) of Ach in eserinized crabs

subjected to sensory stimulation. This not only gives evidence that Ach is involved in sensory transmission, but that it may be found in high concentrations in the general circulation in the presence of an AchE inhibitor. Another study links elevated levels of Ach to the behavioral changes observed in the present study. Sorenson (1973) treated the thoracic ganglion of crabs with Ach (10^{-8} g/ml) by arterial perfusion. This produced a general excitatory effect on the motor neurons and increased the muscle activity. The effect was potentiated by prior infusion with eserine and blocked by atropine.

Release of neurohormones in crustaceans may result from exposure to insecticides, as was seen in insects. Neurosecretory cells containing octopamine and serotonin (5-HT) receive cholinergic innervation in the lobster (Atwood, 1982). Octopamine and serotonin both have excitatory effects on crustacean muscle. Carbaryl-induced release of these neurohormones in particular could lead to hyperactivity, convulsions and paraylsis in crustaceans.

Behavioral changes produced as a result of 1-naphthol intoxication have not been described previously in insects or crustaceans. In <u>in vitro</u> studies 1-naphthol produced mild inhibition of bovine erythrocyte acetylcholinesterase, with an I_{50} (Molar concentration inhibiting 50% of a fixed amount of enzyme after 15 min) of 1×10^{-3} M compared with a value of 5×10^{-8} M for carbaryl (Kuhr and Dorough, 1976). 1-naphthol, therefore, might be expected to produce many of the same behavioral changes as carbaryl but at much higher concentrations, as was seen in this study. The actual differences in the concentrations <u>in vivo</u>

would be affected by the rates of uptake and metabolism of 1-naphthol as compared with carbaryl.

Although Stewart et al. (1967) reported equal sensitivities of male and female ghost shrimp to both carbaryl and 1-naphthol, in the present study larger males appear to be slightly more sensitive to 1-naphthol than do the smaller males or the females. Stewart et al. (1967) did not report whether the males they used were larger than the females but on the average, the ghost shrimp they used were 2.5 times smaller than the ghost shrimp used in the present study. It is possible that they did not test males in this larger size class. They found, however, that male and female Dungeness crabs (Cancer magister) were equally sensitive to carbaryl, but that males were more sensitive to 1-naphthol, although they were the same size as the females.

In summary, both carbaryl and 1-naphthol appear to produce similar signs of nervous system impairment evidenced by hyperactivity, convulsions, and paralysis. 1-Naphthol, however, must be present in concentrations about 50 times higher than carbaryl to produce similar behavioral changes. Although males and females within the range tested appear to be equally sensitive to carbaryl, larger males seem to be the most sensitive to 1-naphthol.

Table 1. Stages of intoxication in ghost shrimp exposed to carbaryl and 1-naphthol.

Stage	Description	Signs
1	Quiescent	Calm, quiet, normal stance maintained
2	Agitated	Active, constantly walking or swimming, normal posture maintained
3	Convulsive	Display constant flexion of the abdomen and appendages; unable to remain upright, remaining on side or back
4	Paralyzed	Resting on side or back displaying only occasional movement; body and appendages flexed
5	Moribund	Resting on side or back displaying no spontaneous movement, weak movement noted only when touched

Table 2. The toxicity of carbaryl to some aquatic arthropods.

Species	24 hr LC_{50} (mg/l)*	Temp. (°C)	Reference
C. californiensis	0.0825 +	12	This study
	0.13	20	Stewart et al., 1967
Hemigrapsus oregonensis	0.49 ++	20	"
Cancer magister	0.0615 ++	20	"
	0.49	10	Buchanan et al., 1970
Gammarus fasciatus	0.05	21	Sanders, 1972
Ascellus brevicaudus	0.320	21	"
Orconectes nais	2.90	21	"
Procambarus clarki	5.0	20	Muncy & Oliver, 1963

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* Includes values reported as TL_m or EC_{50} for irreversible paralysis

\+ EC_{50} for loss of equilibrium

++ Values for males and females averaged

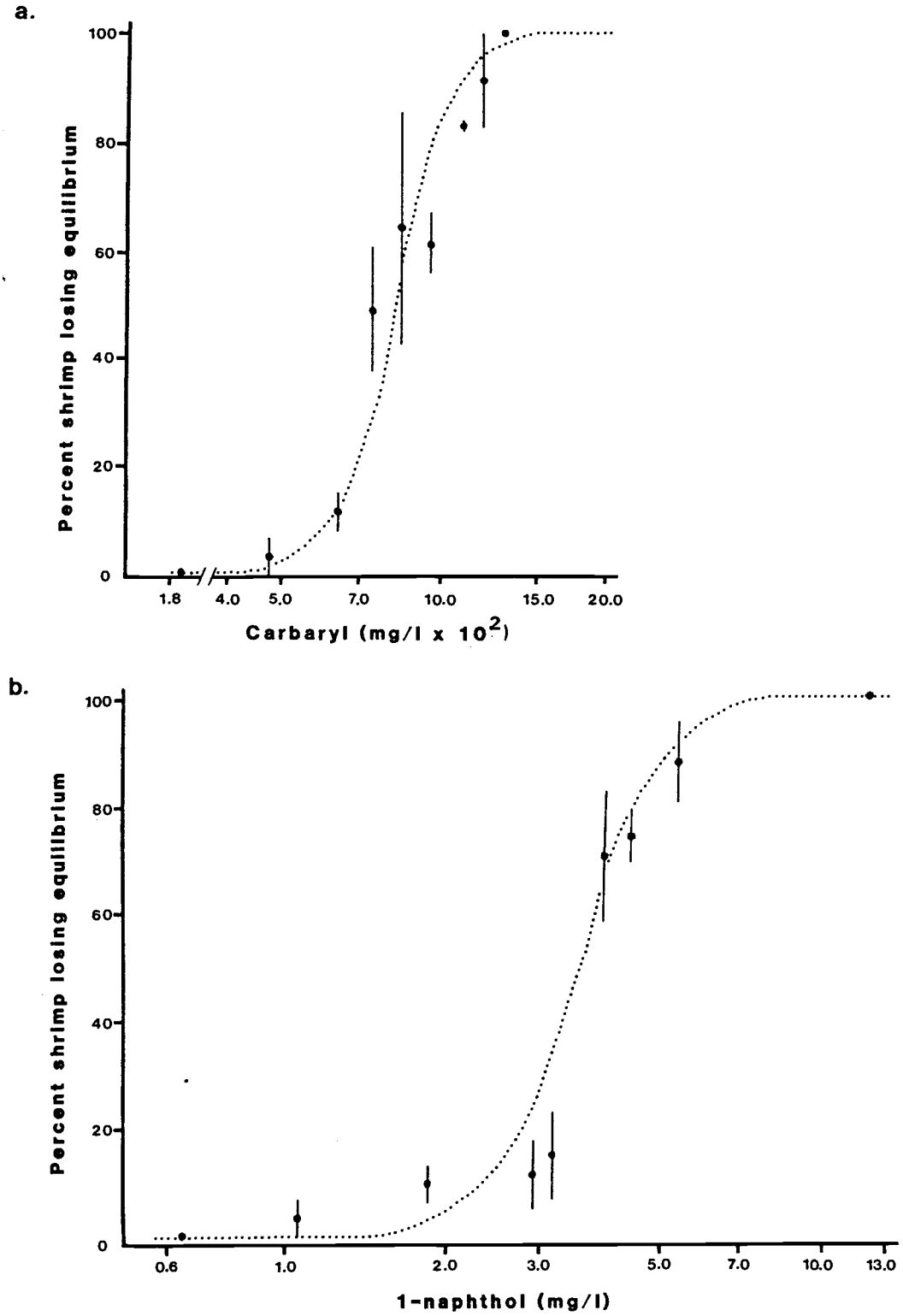

Figure 2. Dose-response curves for ghost shrimp exposed to carbaryl (a) and 1-naphthol (b). Bars represent ± 1 SEM.

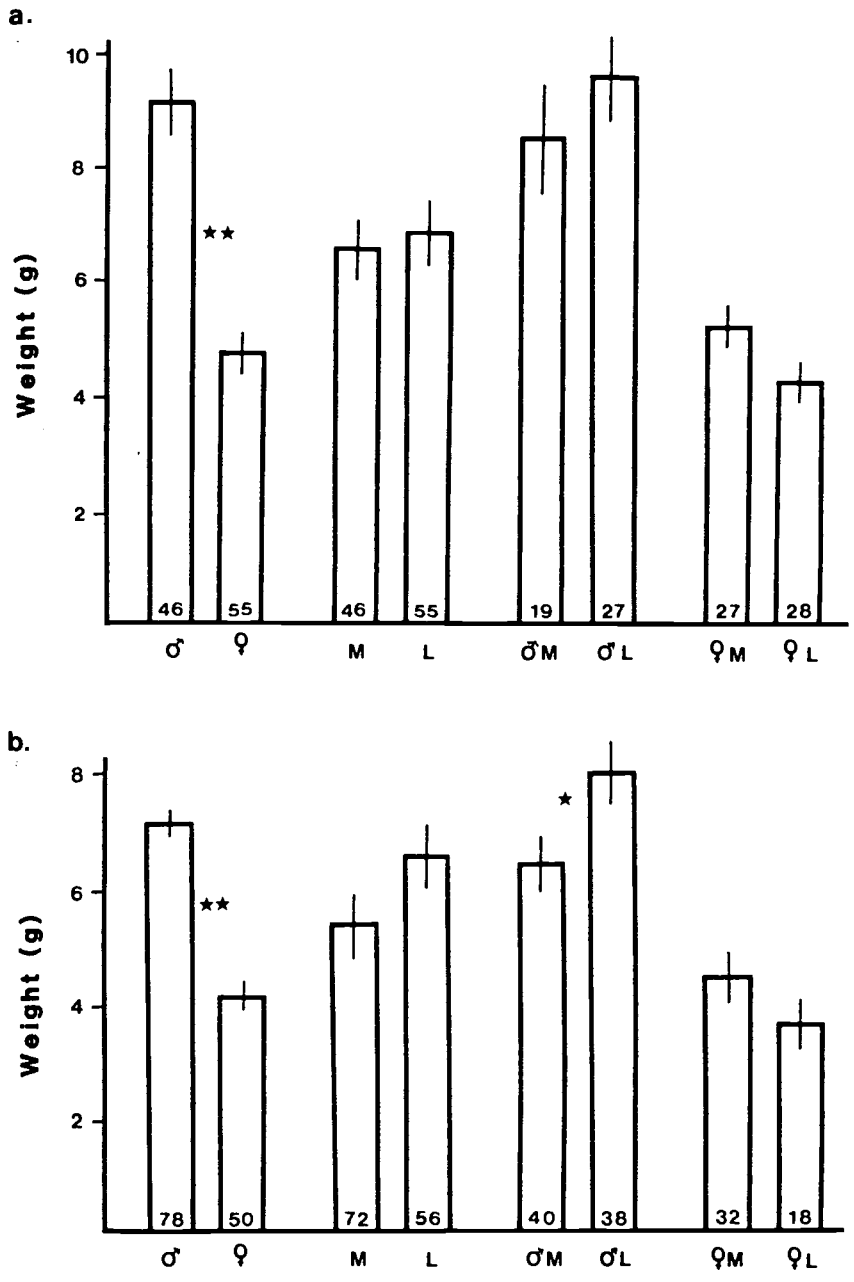

Figure 3. Mean weights of ghost shrimp exposed to carbaryl (a) and 1-naphthol (b), segregated according to sex and response to toxicant. Number of individuals appears at bottom of bar. L = shrimp losing equilibrium during exposure, M = shrimp maintaining equilibrium during exposure. Bars represent ± 1 SEM. (* = $p < 0.05$; ** = $p < 0.01$).

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CHAPTER 2

Routine heart and scaphognathite activity in the ghost shrimp, Callianassa californiensis and changes induced by carbaryl and 1-naphthol treatment

INTRODUCTION

The effect of natural stressors (such as hypoxia, low salinity, high temperature) upon the cardiovascular and respiratory performance of crustaceans has been well-documented (Cameron and Mangum, 1983; McMahon and Wilkens, 1983). The effect of pollution stress upon these two organ systems has received less attention and yet chemical contaminants have become ubiquitous in coastal waters. The studies of Price and Uglow (1980), Baden and Hagerman (1981), Depledge (1981), and Sabourin (1982) have shown that pollutants at sublethal levels can cause distinct changes in the heart and scaphognathite activities of crustaceans. Some bays and mudflats of the Pacific Northwest inhabited by the ghost shrimp, Callianassa californiensis have been subjected to the pesticide carbaryl and its degradation product 1-naphthol (see General Introduction). In this study, the sublethal effects of these two toxicants in ghost shrimp were examined by monitoring the activity of the heart and scaphognathites.

Any assessment of stress-induced modification of cardiovascular and respiratory function makes a basic understanding of the routine activity of these systems necessary. Although heart and scaphognathite patterns have been examined in many of the larger

decapod crustaceans (McMahon and Wilkens, 1983), little is known about the patterns in many of the smaller species including the burrowing ghost shrimp. Refinement of impedance recording techniques (Dyer and Uglow, 1977) have made the study of smaller species possible. In this study, ghost shrimp were monitored under laboratory conditions using impedance recording techniques to document normal patterns of heart and scaphognathite activity. This activity was then compared with that of ghost shrimp exposed to sublethal levels of carbaryl and 1-naphthol.

MATERIALS AND METHODS

Ghost shrimp were collected and maintained in the laboratory as described previously (Chapter 1). Large individuals, 7.4-15.7 g ($\bar{x}$ = 10.6 g), were selected for the experiments as they were easier to implant with electrodes.

Impedance electrodes were prepared from teflon-coated silver wire, diameter 0.005 in. (A-M Systems, Inc.; Everett, WA). The carapace immediately above the heart was dried and a small hole made with an insect pin near the anterior portion of the pericardium. Preliminary recordings indicated that this position yielded high amplitude recording. Two mm of wire, from which the teflon had been removed, was inserted just under the carapace so that it would not interfere with the movement of the heart nor puncture the pericardium. The electrode was sealed in place with cyanoacrylate adhesive (Krazy Glue, Inc., Itasca, IL). Scaphognathite electrodes

were prepared and implanted in a similar manner. The bare tip of the electrode was inserted into a hole made just above the anterior tip of the elevated scaphognathite, as this position yielded single-phase recordings. After the electrode was anchored using cyanoacrylate adhesive, the wire was bent upward toward the midline and glued to the branchiostegite with Scotch super strength adhesive (3M Co., St. Paul, MN) to prevent the thoracic appendages from becoming entangled in the electrodes. A pedestal was glued to the dorsal carapace anterior to the heart to restrain the ghost shrimp during experiments. The pedestal, once clamped to a ring stand, allowed the ghost shrimp to move its appendages and abdomen in walking, swimming, or resting positions, while preventing it from turning around or over.

Each electrode was connected by a pin connector to an impedance pneumograph transducer (Model Mk IV; Narco Bio-Systems, Inc., Houston, TX). The signals from the transducers were amplified and recorded by chart recorders (Model 2200S) with Universal amplifiers (Gould, Inc., Cleveland, OH). A common reference electrode was made of a folded square of aluminum foil (approximately 8 by 6 cm). The experimental set-up is shown in Figure 4.

Prepared ghost shrimp were placed into the experimental chamber which consisted of a 700 ml glass dish containing no sediment. Although Cumberlidge and Uglow (1977) found that the crab Carcinus maenas acclimated to test conditions more rapidly when provided with a natural substrate, McDonald et al. (1977) found that the overall patterns of heart and scaphognathites were not different in crabs

provided with sediment or with a smooth hard surface. Therefore, sediment was not provided due to possible complications arising from the adsorption of toxicants onto the sediment during experiments (Kanazawa et al., 1975). A partial glass divider separated the shrimp from the reference electrode. The seawater within the experimental chamber was kept constantly aerated and at 12° C in a circulating water bath (Gilson Medical Electronics, Middleton, WI). A screen was placed over the chamber to shield the shrimp from visual stimuli; movement and shadows cause transient changes in heart and scaphognathite rhythms in ghost shrimp (Eddy, 1978). Constant illumination was maintained to minimize photoperiod effects; Dyer and Uglow (1978) found that heart and scaphognathite rates of the shrimp Crangon crangon were significantly different during light and dark periods.

Recordings of approximately 2 min duration were made at regular intervals during a 48 hr pretreatment (acclimation) period, a 24 hr treatment (exposure) period, and a 48 hr post treatment (recovery) period for a total of 120 hr (5 days); the recording schedule is shown in Table 3. All shrimp were set up at the same time of day to minimize any confounding effects of circadian rhythms. The seawater in this static system was changed every 24 hr. At the conclusion of the pretreatment period, seawater containing either carbaryl or 1-naphthol was introduced. Control shrimp received a regular water change at this time. At the end of the treatment period the seawater containing toxicant was removed and replaced with toxicant-free water. Preliminary recordings showed that organ rates were elevated

immediately after water changes, but returned to normal within 3-15 min, well before any scheduled recording session.

The EC_{100} level of carbaryl ($\bar{x}$ = 0.148 mg/l) or 1-naphthol ($\bar{x}$ = 6.81 mg/l) was introduced to the chamber at the beginning of the treatment period. The EC_{100} level was chosen so that all animals would reach a similar stage of intoxication during the treatment period. The preparation of the toxicant solutions has been described previously (Chapter 1), except that the stock solution was added to the seawater and the test concentration was determined prior to the placement of the medium into the experimental chamber. Over the course of 24 hr, the carbaryl level declined 45% and the 1-naphthol level declined by 64%.

Heart and scaphognathite rates, and scaphognathite patterns were determined for each recording session. An upward pen deflection represents movement of the organ toward the electrode while a downward deflection represents the organ moving away from the electrode. In the case of the heart, an upward deflection represents systole and a downward deflection diastole (Depledge, 1981). A complete scaphognathite cycle is represented with a single upward and downward deflection when the electrode is placed just above the elevated tip of the scaphognathite. An overall rate for each organ in beats per min (bpm) was determined by counting the total peaks and dividing by the total duration of the recording session. The amplitude of the beats was also noted during each recording session; the amplitude provides at least a relative measure of stroke volume. Depledge (1981) found that changes in impedance trace height do

reflect major variations in stroke volume of crab hearts.

Three distinct scaphognathite beat patterns were classified in the recordings. By counting the total time that the scaphognathite spends in any one pattern and dividing by the total duration of the recording, the percentage of time spent in each pattern was calculated. The scaphognathite beat patterns were also examined by making brief intrabranchial pressure recordings. These recordings were made by placing cannulae into the gill chambers. The cannulae were then connected to pressure transducers. This allowed measurements of intrabranchial pressure to be correlated to the impedance trace. Pressure recordings were calibrated in cm H_2O.

Data was analyzed using one way analysis of variance (ANOVA) and Fisher's least significant difference (LSD) test. Scaphognathite beat patterns reported as percentages were subjected to arc sine (angular) transformation before statistical analysis (Sokal and Rolf, 1967).

RESULTS

I. Normal Heart and Scaphognathite Patterns

The activity of the heart in ghost shrimp can be described in terms of frequency and amplitude of the beats. At one end of the range of activity is the high frequency, high amplitude beating designated tachycardia. At the other end is the low frequency, low amplitude beating designated bradycardia. Changes in frequency are

almost always accompanied by similar changes in amplitude, but this will be described in more detail later in this section. Examples of heart patterns are shown in Figure 5a.

The scaphognathites also show variations in beat patterns attributable to changes in frequency and amplitude. Rhythmic beating can be described in these terms, but two other patterns exist which may only be quantified in terms of their duration. Periods of apnea (cessation of beating) are common and their duration is highly variable. Periods of arrhythmic beating, in which complete scaphognathite cycles cannot be identified, may interrupt periods of rhythmic beating or apnea. Examples of these scaphognathite patterns are shown in Figure 5b.

Effects of the scaphognathite beat patterns on intrabranchial pressure were examined so that changes in ventilatory flow could be assessed. Forward rhythmic beating produces intrabranchial pressures oscillating between 0 and -0.5 cm H_2O. The higher the amplitude and frequency of the beating, the more negative the pressure. Apnea produces an intrabranchial pressure of 0, equal to the external medium (Figure 6a). Beat reversals, rhythmic beating which reverses the direction of the respiratory current, produce positive pressures up to 0.5 cm H_2O (Figure 6b). This pattern which is rarely seen in ghost shrimp, could not be detected using impedance electrodes alone. In most cases, arrhythmic beating produces little change in the intrabranchial pressure and resembles apnea in pressure recordings.

One arrhythmic pattern, termed "clamping", produces momentary

positive pressures up to 1.0 cm H_2O (Figure 6c). Visual inspection of animals correlates this pattern with quick adductions of both branchiostegites. This adduction seems to compress the chamber and perhaps the gills, resulting in the generation of a positive pressure. In the lobster, Homarus americanus, this thoracic movement is preceded by a half beat reversal on the upstroke and is accompanied by an expansion of the gill chamber (Wilkens and McMahon, 1972). It cannot be determined from this study whether the clamping motion is preceded or accompanied by a beat reversal as both could account for the positive pressure. Clamping may act functionally as a reversal, however, since it raises the branchial pressure and would create a brief reversal in the ventilatory flow.

The left and right scaphognathites display an imperfect coordination. Though the scaphognathite frequencies in ghost shrimp are rarely identical, an average difference of only 7.5 bpm indicates that the rates are very similar overall. However, beating on one side may be completely in phase or completely out of phase with the other side. Unilateral beating is rarely seen in ghost shrimp except for very brief periods lasting less than 1 min. Examples of scaphognathite coordination (and lack thereof) are shown in Figure 7.

Rarely is one scaphognathite completely dominant over the other, either in terms of time spent beating or in the frequency of the beating, though a tendency toward dominance can be observed. On the average, one scaphognathite beats at a higher rate in 66% of the

sessions, while the other beats at a higher rate in 29% of the recording sessions. The highest incidence of dominance noted was 81%. Due to the inconsistency of the dominance within individuals and due to the low average difference in the rates between left and right sides, the two rates were averaged for data presentation and analysis.

Heart and scaphognathite activity following electrode implantation

Due to the stress associated with electrode implantation (eg. handling, aerial exposure, elevated temperatures), all ghost shrimp displayed tachycardia and hypernea for several hours after implantation. Post-implantation heart and scaphognathite rates averaged 80 and 110 bpm, respectively, though rates twice as high as these have been recorded. At this time, the heart-to-scaphognathite frequency ratio averaged 0.75. The amplitudes of the heart and scaphognathite beats were at their maximums at this time as well. Although the scaphognathites were engaged in rhythmic beating most of the time, periods of arrhythmic beating accompanied struggling motions of unacclimated ghost shrimp. Clamping activity and beat reversals were often seen during the first few hours after implantation. Apnea was rarely seen in newly-implanted animals.

Heart and scaphognathite activity following acclimation

Figure 8 presents the heart and scaphognathite frequencies from a single ghost shrimp. This animal exhibited features that were common to most of the others studied. As ghost shrimp acclimated to test conditions, heart rate and amplitude both

decreased, most dramatically within the first 9 hr. Generally, a stable heart pattern emerged for each individual within 24 hr post-implantation. From this time throughout the remaining 120 hr of the experiment, a mean heart rate was established at about 70% of the initial post-implantation rate, for an overall mean of 57.3 ± 1.6 bpm, with oscillations of about ± 15 bpm. The amplitude decreased to about 35% of the initial and usually remained very stable. In a well-acclimated ghost shrimp, the heart rhythm consisted of a pattern of bradycardia in which beats of shorter duration were interspersed with beats of longer duration (1-7 sec).

A substantial decrease in scaphognathite rates, to about 30% of the initial post-implantation value, occured within 9 to 24 hr. Oscillations of about ± 30 bpm may be partially accounted for by the normal "burst" pattern of the scaphognathites in a well adjusted individual. Periods of rhythmic beating are regularly interspersed with periods of apnea. Acclimated ghost shrimp may spend over 50% of the time engaged in apnea. Arrythmic beating, including clamping is rare as are beat reversals (less than 10% of the time). In any burst of beating, wide fluctuations were seen in the amplitude, so a decrease in scaphognathite amplitude did not occur with acclimation.

In inactive, unstressed ghost shrimp, the heart-to-scaphognathite frequency ratio is approximately 1.7. Coordination between heart and scaphognathite patterns is not apparent. While the heart beats at a fairly steady rate with no abrupt changes in pattern, the scaphognathites are most often engaged in a variable pattern of beating and apnea. Recordings are

presented in Figure 9 to illustrate the relationship between the rhythms of the heart and one scaphognathite of an individual. Coordination between heart and scaphognathite rates in an individual is shown graphically in Figure 8.

Although the activity of the heart and scaphognathites of an individual may show relatively little variability upon acclimation, the differences in the rates between individuals may be high. Ghost shrimp of the same size and condition may show heart rate differences of 70 bpm and scaphognathite rate differences of 80 bpm at the same stage of acclimation. The reason for these differences is not known, but presumably they fall within the normal physiological range for this species, which may be highly individualistic.

II. Toxicant-Induced Modifications of Heart and Scaphognathite Activity

Effects on the heart

The effect of carbaryl and 1-naphthol treatment upon heart rate is shown in Figure 10. The rates of the control ghost shrimp did not change significantly throughout the period once they became acclimated. During the treatment period, neither the carbaryl nor the 1-naphthol exposed shrimp had heart rates that were significantly different from the controls, though the rates were consistently higher than those of the controls. However, the carbaryl-treated ghost shrimp displayed heart rates significantly

higher than both the controls and the 1-naphthol-treated individuals throughout most of the post treatment period.

In Figure 11, portions of heart recordings from individuals are presented to illustrate changes in the amplitude of the beats during exposure to carbaryl and 1-naphthol. During treatment with the toxicants, the amplitude of the beats was higher than it was during pretreatment, whereas it remained constant in control animals. Amplitude remained elevated in carbaryl-treated animals, but declined slightly in 1-naphthol-treated animals during the recovery period. Changes in amplitude occurred concurrently with changes in frequency.

Effects on the scaphognathites

The effects of carbaryl and 1-naphthol treatment upon scaphognathite rates are shown in Figure 12. The rates of the control ghost shrimp did not change significantly after acclimation to the test conditions. Pretreatment rates of all shrimp were similar, but toxicant treatment induced immediate increases in the scaphognathite rates, though these increases were statistically significant only during carbaryl exposure. The scaphognathite rates decreased during the recovery period so that during the latter part of this period, the rates were not statistically different from the controls, though they were still elevated in the carbaryl-treated group.

The amplitude of the scaphognathite beats showed no consistent changes throughout the experiment either within treatment groups nor between them (Figure 13). All of the groups displayed the greatest

regularity in amplitude during immediate post-implantation when it was usually elevated. Changes in amplitude did not seem to directly correspond to changes in rate as was seen with the heart beats.

Figure 14 illustrates the relative portion of time engaged in rhythmic beating, arrhythmic beating, and apnea, throughout the experiments. No significant changes were seen in the time spent in any of these patterns in control animals after acclimation. 1-Naphthol-exposed ghost shrimp tended to spent a greater portion of the time engaged in rhythmic beating than did controls during the treatment period. This trend continued until the latter half of the post treatment period. These changes were not statistically significant, however. In the carbaryl treatment group, the amount of time shrimp engaged in rhythmic beating increased significantly ($p<0.01$) during the treatment period and remained elevated for the entire post treatment period ($p<0.01$, compared with pretreatment). Time engaged in rhythmic beating did decline significantly ($p<0.01$, compared with treatment period) during the last half of the post treatment period. The amount of time engaged in arrhythmic beating did not vary significantly during the course of the experiment in any of the treatment groups.

Relationship between heart and scaphognathite frequencies

Acclimated ghost shrimp in all three treatment groups displayed similar heart-to-scaphognathite frequency ratios with the heart rate exceeding that of the scaphognathites by approximately 1.7-to-1 (Table 4). During treatment with 1-naphthol, the ratio changed to about 1-to-1 and during carbaryl treatment, the

scaphognathite frequency exceeded that of the heart by 1-to-1.6. The latter resembled that seen in newly-implanted ghost shrimp. During the post treatment, the ratio in the 1-naphthol-exposed group returned to the pretreatment value, but this recovery was not noted in the carbaryl-exposed group. The scaphognathites continued to beat at a greater frequency than the heart until the latter half of the post treatment period, when the ratio was about 1-to-1.

DISCUSSION

I. Normal Heart and Scaphognathite Activity

Resting heart frequencies of decapods maintained at 12-15° C generally lie between 33 and 100 bpm (McMahon and Wilkens, 1983). Ghost shrimp with mean heart rates of 57 bpm (at 12°C) fall into the middle of this range, eventhough they are one of the smallest species for which rates have been determined. Resting heart patterns which include bradycardia, tachycardia, and cardiac arrest have been reported for Cancer magister (McDonald et al., 1977), Carcinus maenas (Cumberlidge and Uglow, 1977) Homarus americanus (McMahon and Wilkens, 1972) and previously for ghost shrimp (Eddy, 1978). Oscillations of approximately 20 bpm about the mean were common for these species. In the present study, ghost shrimp displayed oscillations of 15 bpm about the mean. Mild bradycardia dominated the heart pattern; tachycardia and prolonged cardiac arrest were never observed in quiet individuals.

Scaphognathite rates, covering a broad range of 50 to 400 bpm, have been reported for crustaceans in a variety of circumstances (McMahon and Wilkens, 1983). High individual variation in scaphognathite beat frequencies may be accounted for by the "burst" pattern of beating common in many crustaceans. This pattern consists of intervals of rhythmic beating separated by intervals of apnea. In resting lobsters, pauses of 3-8 min are common (Wilkens and McMahon, 1972). Eddy (1978) reported apneas lasting up to 40 min in unstressed ghost shrimp. In ghost shrimp, the scaphognathite pauses are most often bilateral as they are in the shrimp Crangon crangon (Dyer and Uglow, 1977), but in many crustaceans unilateral pauses are more common (Taylor, 1982).

Apnea may decrease the cost of ventilation during periods of low oxygen demand. Evidence suggests that scaphognathite frequencies below 30-40 bpm may not efficiently ventilate the branchial chamber due to the backflow of water between beats (Taylor, 1982; Burnett and Bridges, 1981). Bilateral scaphognathite bursts punctuated by apnea may therefore meet the oxygen demands of a resting animal at lower energetic cost than slow steady scaphognathite beating. Likewise, unilateral pumping may be an efficient way of meeting the oxygen demands of a resting animal (Wilkens, 1981; Taylor, 1982).

Reversal beating is often seen in resting crustaceans, but the role of these reversals in normal activity is often not clear. Resting lobsters, for example, typically display halt beat reversals (Wilkens and McMahon, 1972). Branchial water flow is reduced during

reversals, and so may accompany low oxygen demand (McMahon and Wilkens, 1983). Sustained beat reversals are seen most commonly in burrowing brachyurans such as Cancer magister (McDonald et al., 1977), but have also been seen in a few macrurans such as Crangon crangon (Dyer and Uglow, 1978). Beat reversals, in burrowing species which maintain their anterior respiratory channel above the sediment surface, may insure a clean supply of water to the gills. Beat reversals were not observed in resting ghost shrimp in this study. Although ghost shrimp are burrowers, they live in impermanent burrows completely below the surface, so reversals would not insure a sediment-free respiratory current. Instead, constant grooming with setose appendages (MacGinitie, 1934) may serve to remove sediment particles from the gill chamber. In an hypoxic environment (Thompson and Pritchard, 1969), where oxygen uptake would have to be maximized, ventilation-reducing beat reversals might not be advantageous.

Although the amplitude and so probably the stroke volume of the heart beats of acclimated ghost shrimp showed little variation (10%), greater variability was noted in the scaphognathite beat amplitude. Typically, any single burst of beating contained beats of various amplitudes. Before measurements of branchial water flow were actually made, scaphognathites were thought to be fixed volume pumps (Wilkens, 1981; McMahon and Wilkens, 1983), but the direct relationship between frequency and branchial water flow is no longer supported. In ghost shrimp, changes in frequency seem to affect intrabranchial pressures as much or more than changes in the

amplitude, although the relative contributions of each were not investigated.

The degree of coordination between the heart and scaphognathites varies from species to species. In lobsters, either the two scaphognathites or the heart and one scaphognathite beat in phase, but never all three (Wilkens, 1981). In the crabs Cancer borealis and C. irroratus the heart and one scaphognathite beat in phase 98% of the time while the other scaphognathite displays an independent pattern (Coyer, 1979). In most decapods, including ghost shrimp, the scaphognathites seem to be the more highly coordinated (McMahon and Wilkens, 1983). In ghost shrimp, only small differences occur between left and right scaphognathite beat frequencies, but the resting heart-to-scaphognathite frequency ratio is 1.5. Coordination was most noticable between heart and scaphognathites during long ventilatory pauses in which the heart rate gradually became slower. When demands for oxygen are low, bradycardia and apnea are often seen concurrently in crustaceans (Wilkens, 1981).

II. Heart and Scaphognathite Activity Following Electrode Implantation

During this study, the heart rates of newly-implanted ghost shrimp were elevated 30% over resting rates while the amplitude was 65% above normal. In general, it took over 9 hr for the heart rate and amplitude to decrease and stabilize. Eddy (1978) reported elevations in heart rate of 40 to 80 bpm, lasting up to 15 hr after

electrode implantation. Elevated heart rates lasting over an hour have been reported for Carcinus maenas subjected to mild disturbance (Cumberlidge and Uglow, 1977). As was seen in this study, McMahon and Wilkens (1983) report that disturbances often result in a much greater increase in stroke volume than in frequency in several decapod species. In some cases, the stroke volume increased 2 to 4.3 fold while the frequency remained essentially constant. Stroke volume, therefore may be very important in increasing cardiac output (ml per kg per min) in stressed crustaceans; the crustacean heart may simply be incapable of large changes in frequency (Cameron and Mangum, 1983).

In this study, average scaphognathite rates of 110 bpm were recorded immediately after electrode implantation. This represents a 70% increase in rate compared with acclimated animals. Disturbed crustaceans often display high frequency forward beating which may be elevated 6 fold above resting levels (Wilkens, 1981). In the crab Carcinus maenas, post-implantation rates as high as 400 bpm were observed though the average rate was 120 bpm. In these crabs, resting scaphognathite rates were not achieved for 48 hr after implantation and with the decrease in rate came a decrease in the occurrence of reversals as well (Cumberlidge and Uglow, 1977). Incidences of reversal beating and clamping were common in newly-implanted ghost shrimp, though these patterns were almost never observed in acclimated animals. These patterns have been reported in lobsters and crabs returned to water from the air (Wilkens and McMahon, 1972; Berlind, 1977) and may represent

attempts by the animals to rid their gill chambers of air. During scaphognathite electrode implantation in this study, small air bubbles were often introduced into the gill chambers. Clamping behavior may be an attempt to rid the chamber of the bubbles or perhaps the electrodes.

There are several reasons why heart and scaphognathite performance might be elevated following electrode implantation. Emersion of the ghost shrimp during electrode implantation may have depleted oxygen stores and resulted in a reliance on anaerobic metabolism. Pritchard and Eddy (1979) have confirmed the ability of ghost shrimp to accumulate lactate in hypoxic situations. It took 6 hr for lactate levels to return to normal in the crab Cardisoma carnifex after forced exercise (Wood and Randall, 1981). Sensory stimulation or stress may trigger massive releases of neurohormones in crustaceans (J.L. Wilkens, pers. com.). Serotonin (5-HT), a monoamine neurohormone increases the rate and force of contraction of the heart and the rate of the scaphognathites (Wilkens, 1981). Serotonin also increased the occurrence and duration of scaphognathite reversals in Carcinus maenas (Berlind, 1977). The release of hormones and oxygen debt may both be able to explain the long term changes in heart and scaphognathite patterns seen in newly-implanted ghost shrimp.

III. Toxicant-Induced Changes in Heart and Scaphognathite Activity

The insecticide carbaryl, and to a lesser extent its

degradation product 1-naphthol, produced changes in heart and scaphognathite activity in ghost shrimp at sublethal doses. No other studies have examined the effects of insecticides upon heart and scaphognathite activities in crustaceans. Although the proposed mode of action of carbaryl, and perhaps 1-naphthol, is the reversible inhibition of acetylcholinesterase, nonspecific interactions of these two compounds acting simply as foreign compounds may affect the activities of these organs.

Nonspecific interactions

The changes noted in heart and scaphognathite activity could be caused by a reaction of the ghost shrimp to a novel chemical stimulus. Novel stimuli often affect heart and scaphognathite activities in crustaceans (McMahon and Wilkens, 1983), the most common reaction being bradycardia accompanied by apnea (Dyer and Uglow, 1977; McDonald et al., 1977; Wilkens et al., 1974; Eddy, 1978). Ghost shrimp in the present study did not react to the presence of the toxicants with bradycardia and apnea. One explanation for this would be that they could not detect the toxicants. Pearson and Olla (1980) found that blue crabs could detect very low concentrations of naphthalene. However, Hansen et al. (1973) showed that the grass shrimp Palaemonetes pugio was unable to detect and avoid carbaryl and several other pesticides even at concentrations that would be toxic to them. Another explanation of the reaction of the ghost shrimp would be that they could detect the toxicants, but that they reacted with an escape response rather than with bradycardia and apnea. Pearson and Olla

(1980) found that prolonged presence of perceived chemicals often leads to an escape response which may be associated with increased in heart and scaphognathite activity (Saborin, 1982). Therefore, the increases in the activity of the organs seen in ghost shrimp could be part of an avoidance response.

Since changes in the scaphognathite activity were the most dramatic, carbaryl and 1-naphthol may be acting simply as gill irritants, causing the ghost shrimp to increase the ventilatory flow. If this were the case, then many pollutants such as oil and heavy metals might cause this response by depositing on the gill surface.

Price and Uglow (1980) exposed Crangon crangon to the heavy metals cadmium (Cd), copper (Cu), and zinc (Zn). During a 48 hr exposure to 20 mg/l of each metal, the shrimp displayed steady increases in scaphognathite and heart rates, accompanied by increased activity. The Cu and Zn treated animals had a high rate of beat reversals and precipitation of the metals onto the gills was noted. At concentrations of 1.0 and 5.0 mg/l, rates were elevated but returned to normal by the end of the 48 hr in Cu and Zn exposed individuals. Depledge (1981) examined the effects of Cu and mercury (Hg) upon the heart rate of Carcinus maenas. The rate decreased and then became highly variable in response to Cu. Treatment with Hg led to a 40-90% decrease in heart rate associated with elevated stroke volume.

Depledge (1981) also examined the effects of the water soluble fractions (WSF) of crude oil and an oil dispersant upon heart and

respiration rate ($\mu l\ O_2/g/hr$) of Carcinus maenas. Only the dispersant alone caused a significant increase in both rates within 15 minutes, but rates returned to normal within 24 hr. Sabourin (1982) exposed Callinectes sapidus to naphthalene, a water soluble component of crude oil. Although bradycardia and apnea were common in control animals, they were abolished in experimental animals. Heart and respiration rates were elevated at the higher dose tested. Baden and Hagerman (1981) studied the long term effects of crude oil WSF on the scaphognathite pattern of Palaemon adspersus. During a two week exposure period, time spent engaged in arrhythmic beating increased, sometimes as high as 100%. Recovery was noted in almost all individuals within 5 weeks.

The response of crustaceans to pollutants seems to be quite variable. This may be just a result of differences in test concentrations, conditions, and species. It is interesting to note that even though 1-naphthol is very similar to carbaryl structurally that concentrations 50 times higher produced less of a response. If both were acting as simple gill irritants, it would seem logical that 1-naphthol would have produced a greater response at that concentration.

Interaction of toxicants with acetylcholinesterase

Carbaryl toxicity may be a direct result of its ability to inhibit AChE at cholinergic synaptic junctions. The inhibition of AChE would cause a build up of ACh at those junctions and would lead to prolonged stimulation of postsynaptic tissues. In order to understand how insecticide treatments would affect heart and

scaphognathite function, a basic understanding of the action of ACh upon the heart and scaphognathites would be necessary.

Few studies have examined the direct effects of Ach and inhibitors of AChE upon the crustacean heart and no studies have examined effects of direct application to the scaphognathites. Davenport (1941, 1942) tested the effects of Ach and the AchE inhibitor eserine (physostigmine) on isolated hearts of Cancer magister which had the cardiac ganglion intact. Low (10^{-6} g/ml) doses of Ach increased both the amplitude and the frequency of the beats. Ach applied after eserine had an even greater excitatory effect. Miller and Kennedy (1973) examined the effect of carbofuran, a carbamate, upon the heart rates of flies. In control flies, heart rate was variable, marked with periods of bradycardia, cardiac arrest, and tachycardia. During topical treatment of intact flies with the insecticide, the heart rate became elevated and regular. In addition, McFarlane and Fong (1972, cited in Miller, 1974) found that eserine potentiated the Ach-induced increase in heart rate in the cricket Acheta domesticus. Although the hearts of crabs and insects were stimulated by Ach and/or AChE inhibitors in the studies mentioned above, no naturally occurring cholinergic synapses have been found in the hearts or cardiac ganglia of crustaceans (Maynard, 1971) or insects (Pichon, 1974). Therefore, although carbaryl and 1-naphthol treatment had a slight stimulatory effect upon the hearts of ghost shrimp, they probably do not act directly at cholinergic synapses in the heart itself.

Evidence suggests that the crustacean heart and

scaphognathites may be influenced by Ach through the CNS. The cardioaccelerator fibers from the subesophageal ganglion are stimulated by applied Ach (Taylor, 1982). Ach, perfused into the thoracic ganglion of Carcinus maenas where the scaphognathite oscillators are located (Berlind, 1977), caused a small increase in the incidence of beat reversals. Ach did not cause an increase in rate, however.

Ach may also influence heart and scaphognathite activity indirectly by stimulating the release of neurohormones from neurosecretory cells. In insects, the release of neurohormones has been seen in response to insecticides which increase Ach levels (Maddrell and Reynolds, 1972). Singh and Orchard (1982) found that neurosecretory cells were more sensitive to insecticides than were other nerve cells, and that neurohormone release actually preceded nervous system hyperactivity in insects. Neurosecretory cells located along the second root of the thoracic ganglion in lobsters receive cholinergic innervation (Atwood, 1983) and release serotonin and octopamine when stimulated. Both of these hormones have been found to influence heart and scaphognathites activities (Wilkens, 1981; Berlind, 1976, 1977). There is a distinct similarity between heart and scaphognathite rhythms following electrode implantation and following carbaryl and 1-naphthol exposure. Neurohormone release due to the stress of handling and due to insecticide exposure could explain the similarity of these two reactions.

Carbaryl and 1-naphthol may also influence heart and scaphognathite activity indirectly by stimulating skeletal muscle

activity. The neuromuscular junctions of crustaceans are not cholinergic, but cholinergic synapses are located centrally. Perfusion of crab thoracic ganglia with Ach increased muscular activity through a stimulatory effect on the motor neurons (Sorenson, 1973). The reaction of the heart and scaphognathites would then be similar to that seen during strenuous muscle activity. In several crustacean species, cardiac output more than doubles in response to strenuous activity (McMahon and Wilkens, 1983), but this does not have to imply an increase in heart rate. McMahon et al. (1979) found that heart rate increased very little in active Dungeness crabs and Herried et al. (1979) found that heart rate actually decreased in exercising land crabs. Changes in stroke volume may contribute more to changes in cardiac output than does rate (Wilkens, 1981). In the present study, the amplitude of the heart beats increased during toxicant exposure. This may explain why a greater change in rate was not observed.

Toxicant-induced increases in muscle activity could have also produced an oxygen debt which would explain why the scaphognathite and heart rates remained elevated after carbaryl exposure had ended. The carbamate insecticide Pyrolan caused a drop in muscle pH during convulsions in Periplaneta americana which was attributed to the build up of lactic acid (cited in Casida, 1963). Several studies indicate that lactic acid levels do not decrease in crustaceans for at least 24 hr following activity or disturbance (McMahon and Wilkens, 1983). In ghost shrimp 1-naphthol treatment increased the muscle activity just as much as carbaryl treatment, but neither

heart nor scaphognathite rates were elevated significantly over the rates of resting control animals as they were in carbaryl-treated animals.

1-Naphthol intoxication did not result in statistically significant increases in heart and scaphognathite rates, nonetheless they were elevated and therefore similar to those produced by carbaryl intoxication. Variability in the response may account for the lack of statistical significance. It is likely that carbaryl and 1-naphthol affect the heart and scaphognathites in a similar manner, since the behavioral symptoms of intoxication are so similar (see Chapter 1). The response produced by 1-naphthol does seem to be more short-lived, and recovery seems to be faster. This may be due to the ability of the ghost shrimp to metabolize and excrete 1-naphthol at a higher rate than carbaryl or may be due to a decreased mobility across membranes because of its polar hydroxyl group. Most importantly, carbaryl has been found to be a much better <u>in vitro</u> inhibitor of AchE than is 1-naphthol and this may account for the greater magnitude of the response in carbaryl-treated shrimp.

Table 3: Hourly schedule of recording sessions. (WC designates a water change made immediately after the recording session)

Pretreatment period (hr)	Treatment period (hr)	Post treatment period (hr)
1	1	1
3	3	3
6	6	6
9	9	9
21	12	21
24	18	24
WC	21	WC
28	24	28
32	WC	32
44		44
48		48
WC		

Table 4: The ratio of heart-to-scaphognathite frequencies.

Period @	n	Control		1-naphthol		Carbaryl	
IMPLT	2	0.72=0.05		0.65=0.01	*	0.85=0.01	*
ACCLM	5	1.80=0.23		1.58=0.08		1.79=0.27	
TMT	8	1.58=0.11	**	1.00=0.07	**	0.61=0.06	**
POST 1	6	1.51=0.12		1.30=0.03	**	0.80=0.07	**
POST 2	4	1.53=0.14		1.69=0.17	**	1.07=0.05	*

@ IMPLT: 1-3 hr post-implantation, ACCLM: 24-48 hr pretreatment, TMT: entire treatment period, POST 1 and 2: first and second 24 hr post treatment periods.

* $p < 0.05$, ** $p < 0.01$. Stars placed between treatment columns refer to the difference between those treatments. Stars placed after the carbaryl values signify difference from control values.

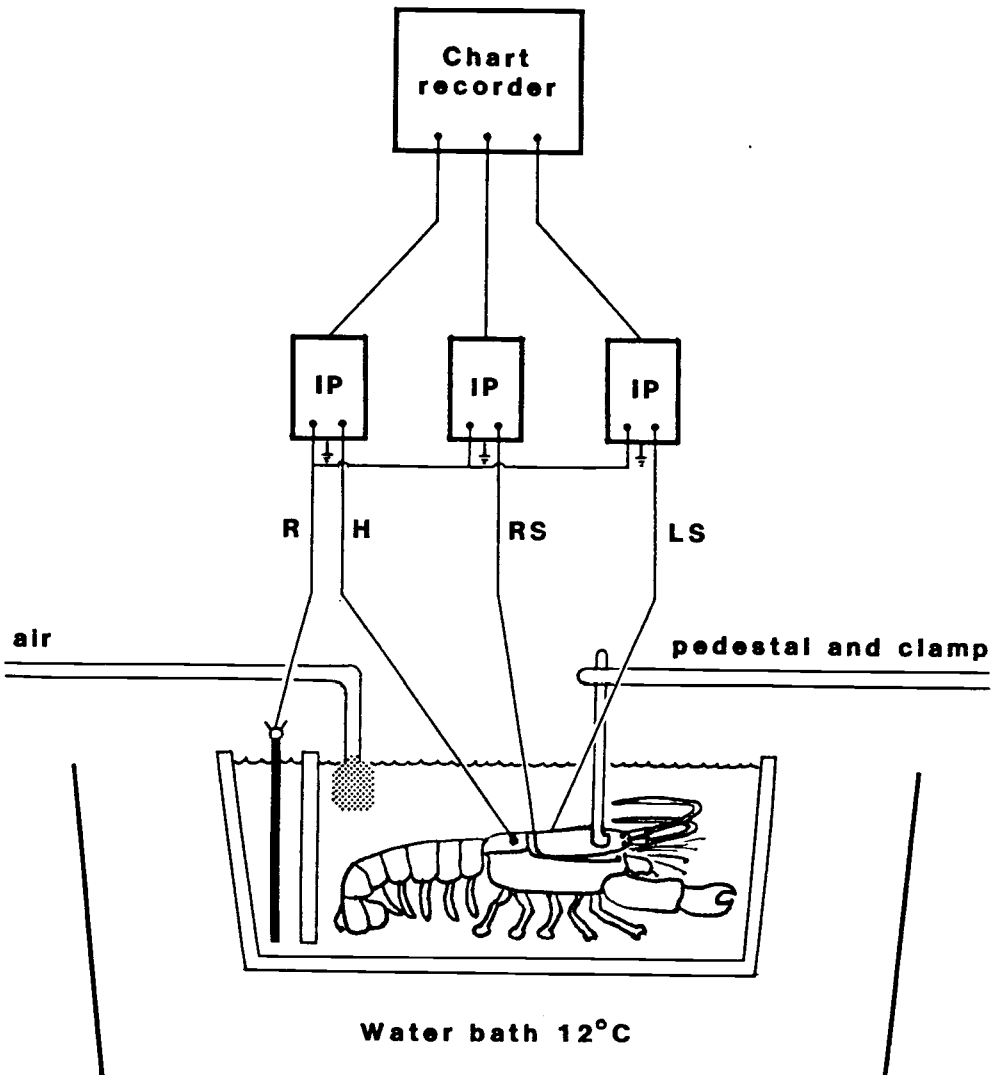
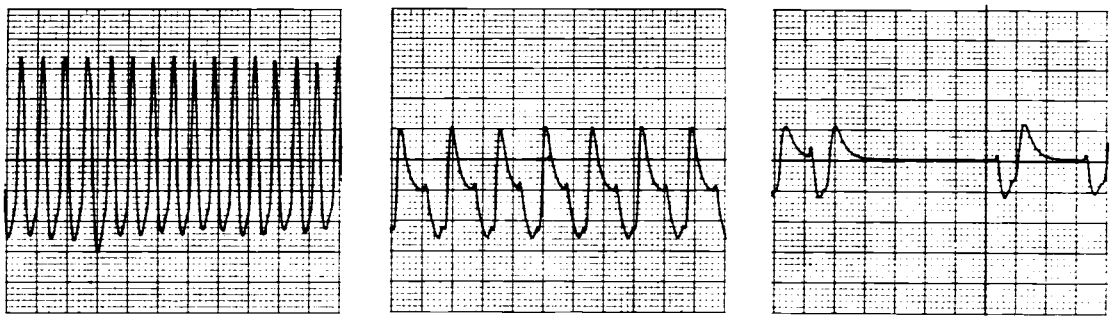


Figure 4. Experimental set-up for monitoring heart and scaphognathite patterns in ghost shrimp (after Dyer and Uglow, 1977). IP = impedance pneumographs, R = reference electrode, H = heart electrode, RS = right scaphognathite electrode, LS = left scaphognathite electrode.

a.



b.

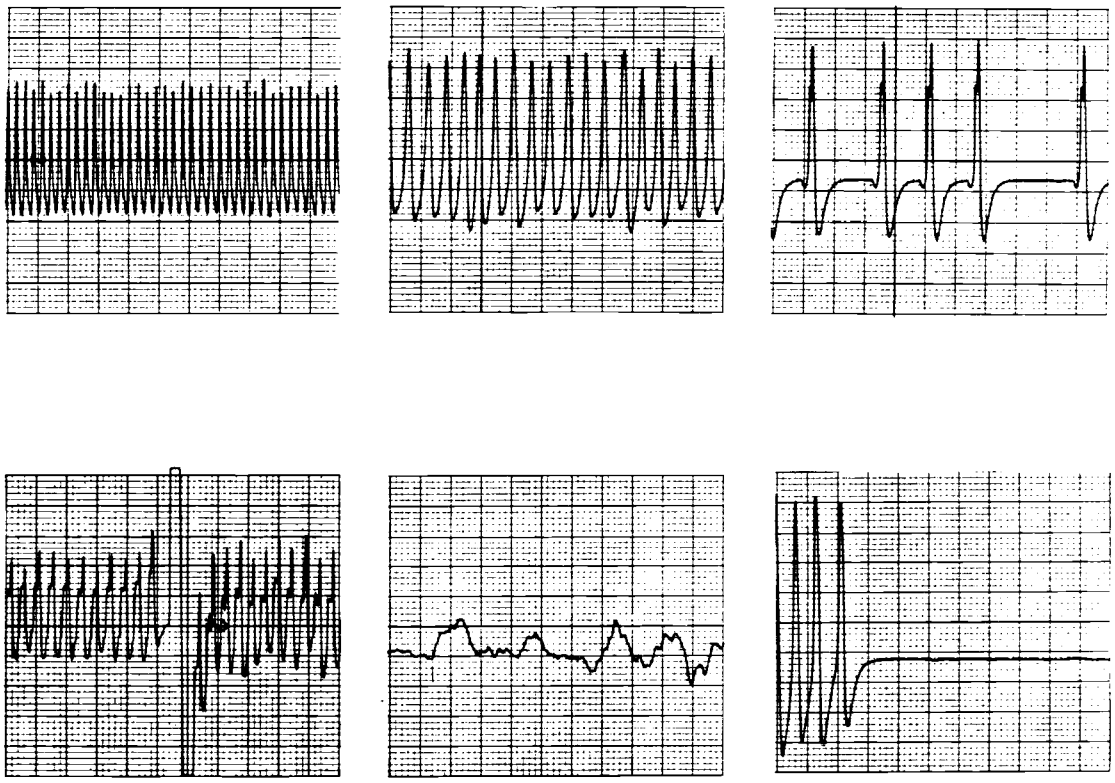


Figure 5. Examples of beat patterns of the heart (a) and scaphognathites (b) in ghost shrimp.

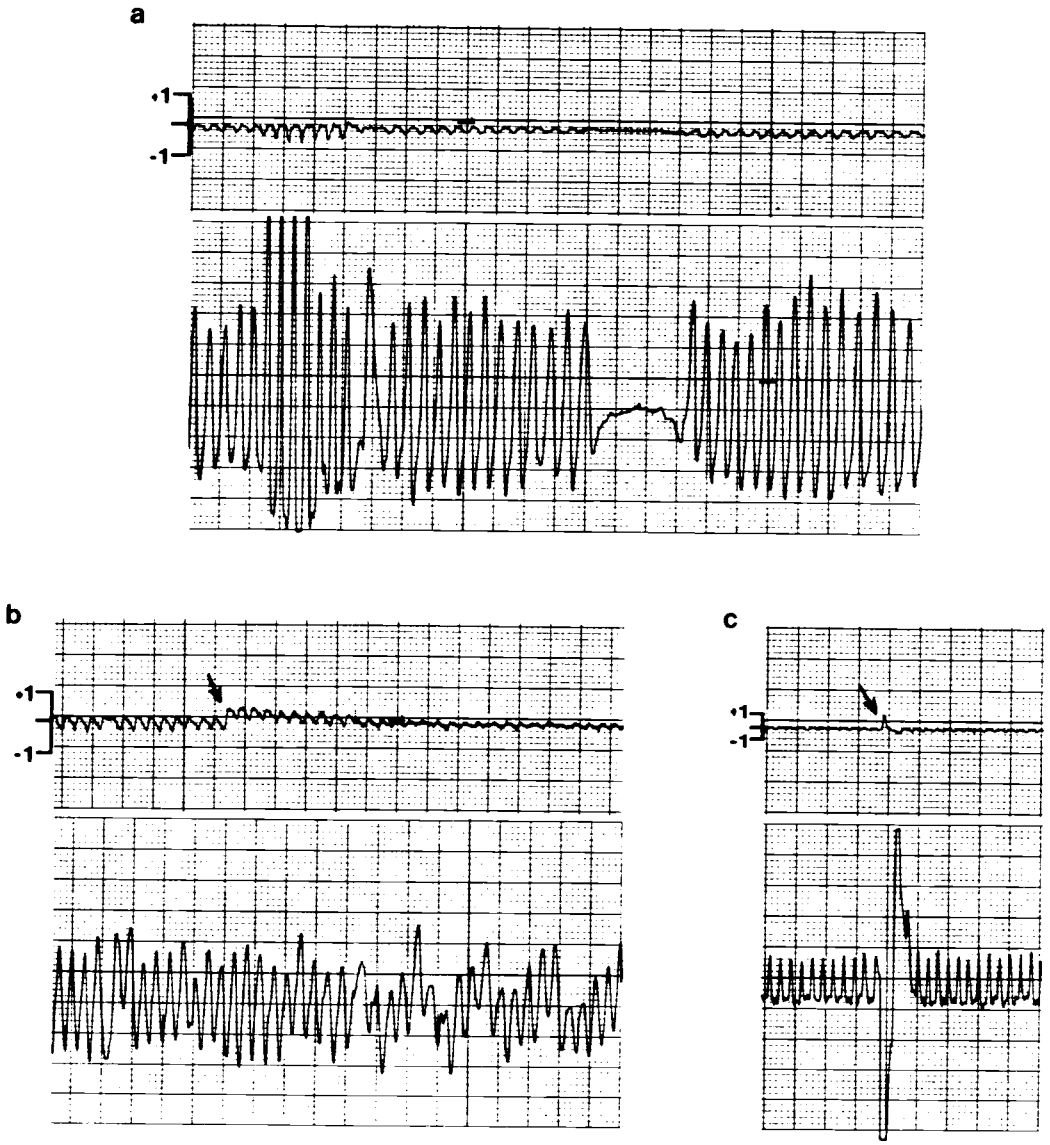


Figure 6. Recordings of intrabranchial pressure (top trace) made simultaneously with impedance recordings (bottom trace) during different scaphognathite beat patterns. Patterns include variations in amplitude and rate (a), reversal beating (b) and clamping (c). Pressure calibration is ± 1 cm H_2O.

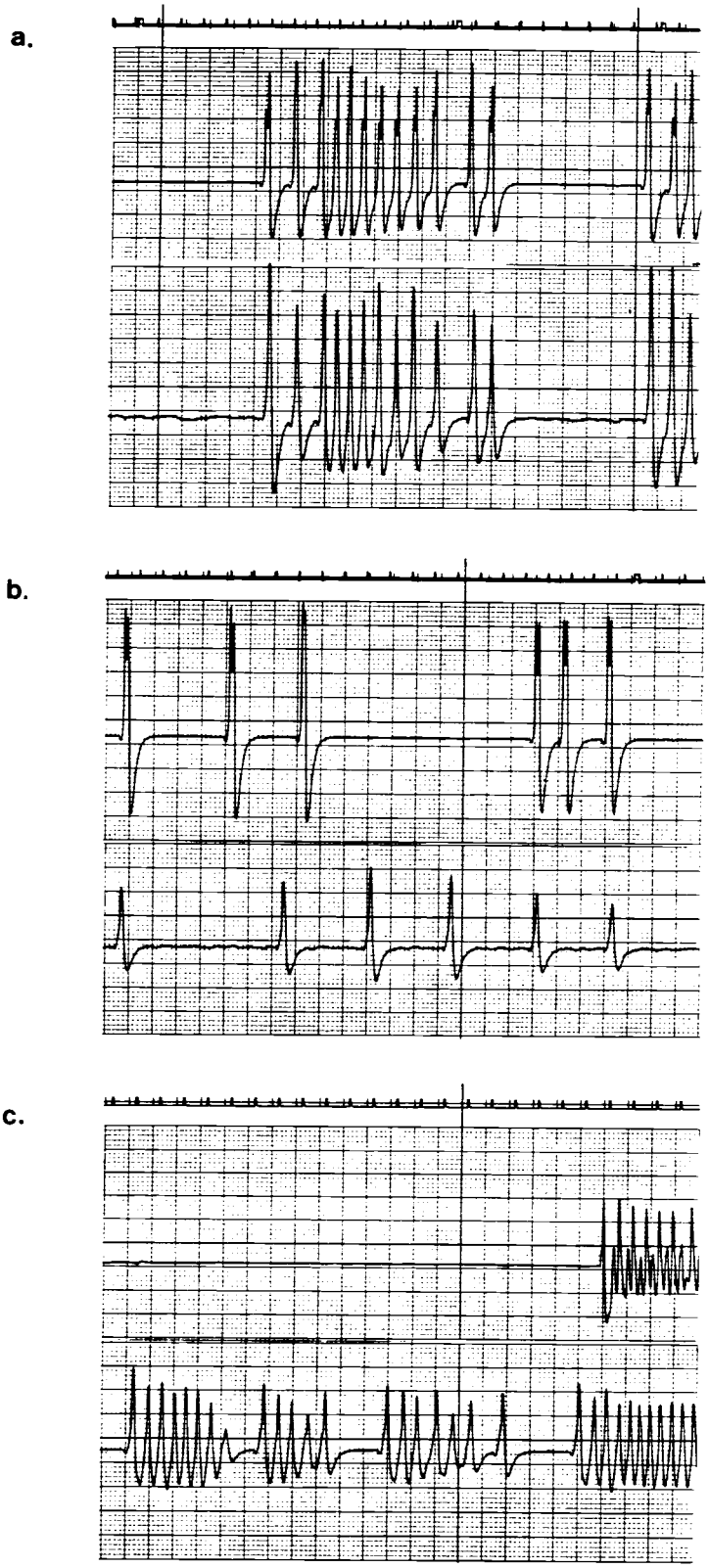


Figure 7. Patterns of coordination between left and right scaphognathite, showing phase coordination (a), phase drift (b) and unilateral beating (c). (Recordings from different individuals.)

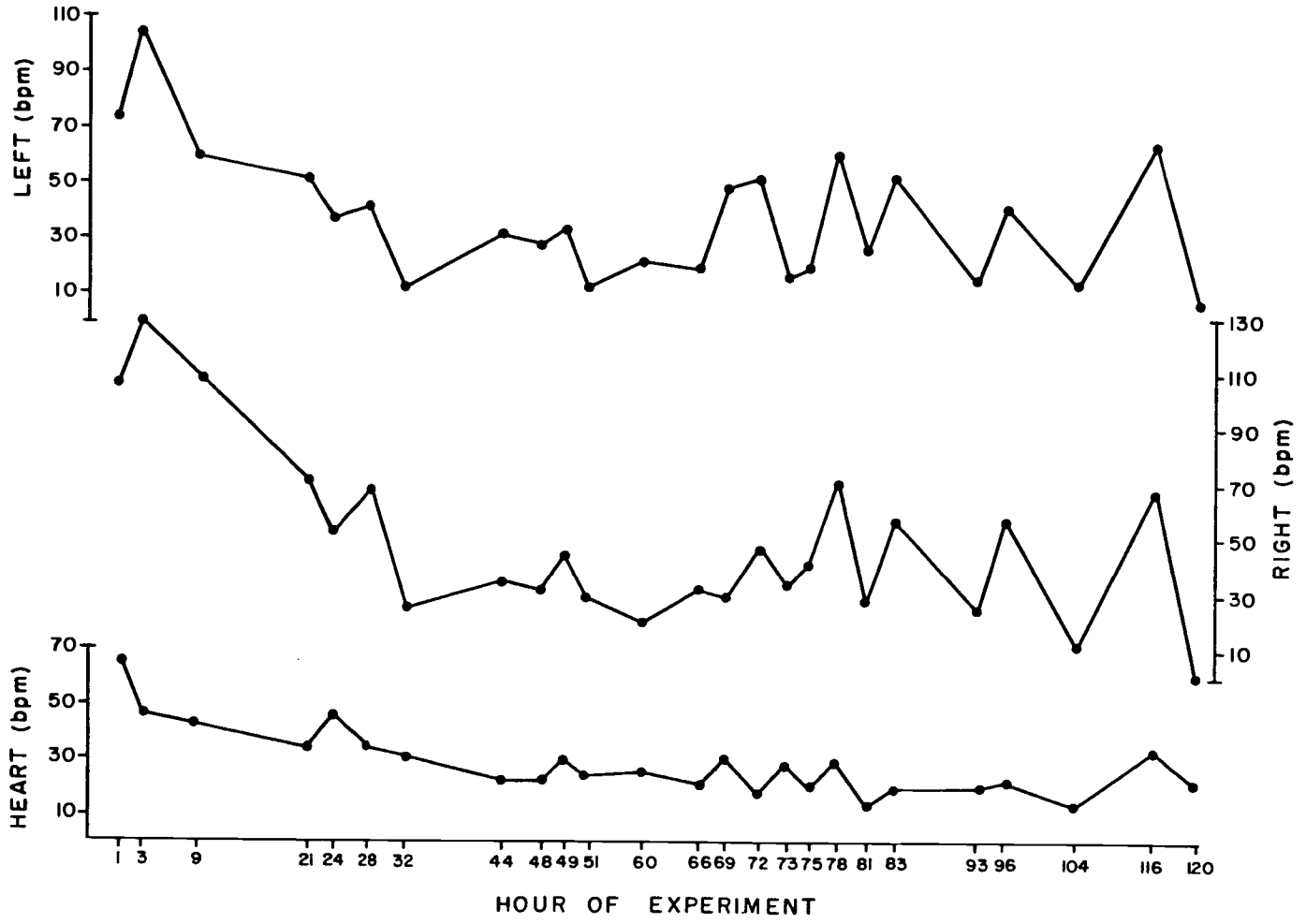


Figure 8. Heart, and left and right scaphognathite frequencies of an individual control ghost shrimp throughout 120 hr of monitoring.

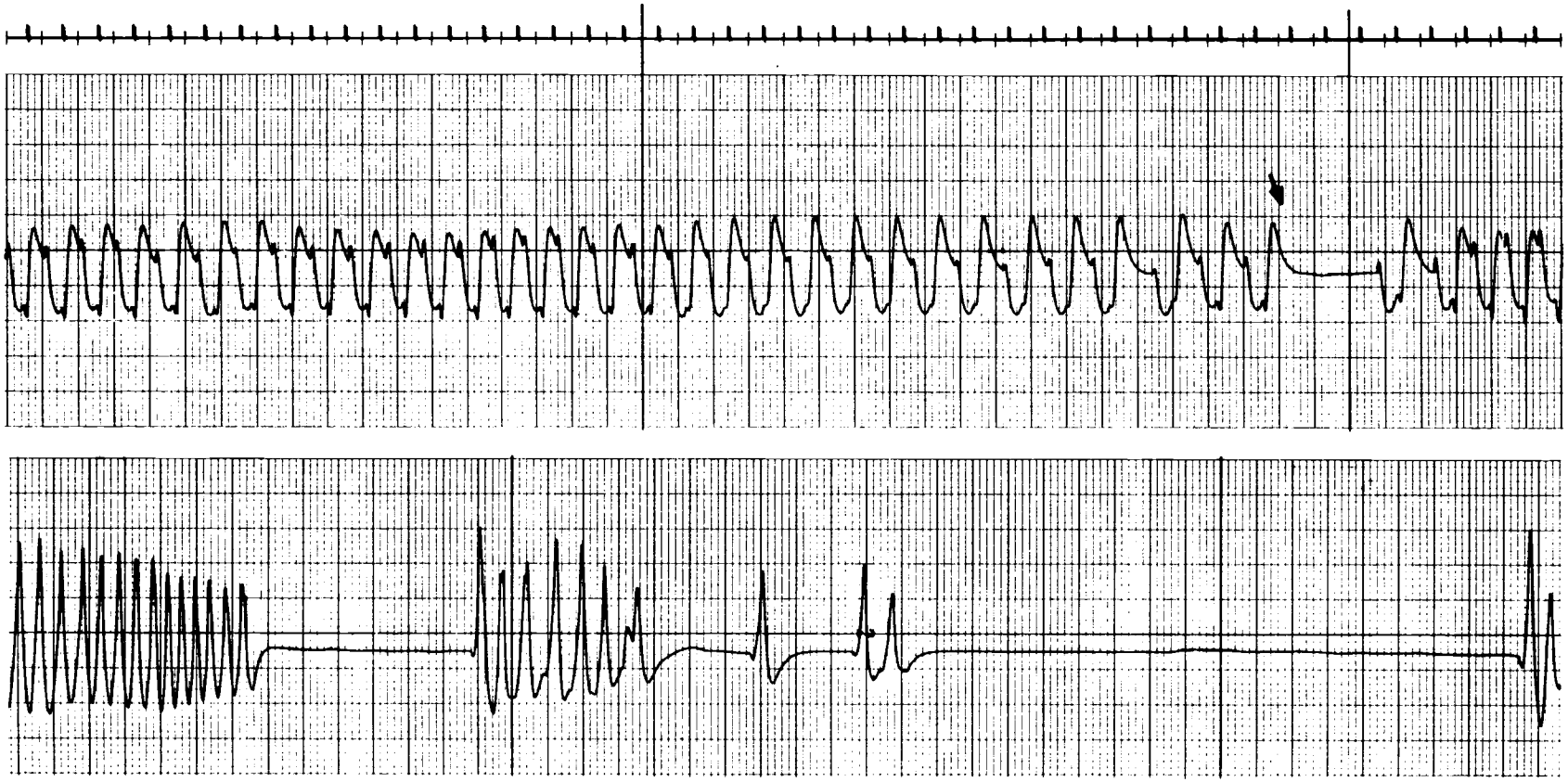


Figure 9. Example of coordination between the heart and one scaphognathite in an acclimated ghost shrimp. Arrow indicates brief bradycardia associated with apnea.

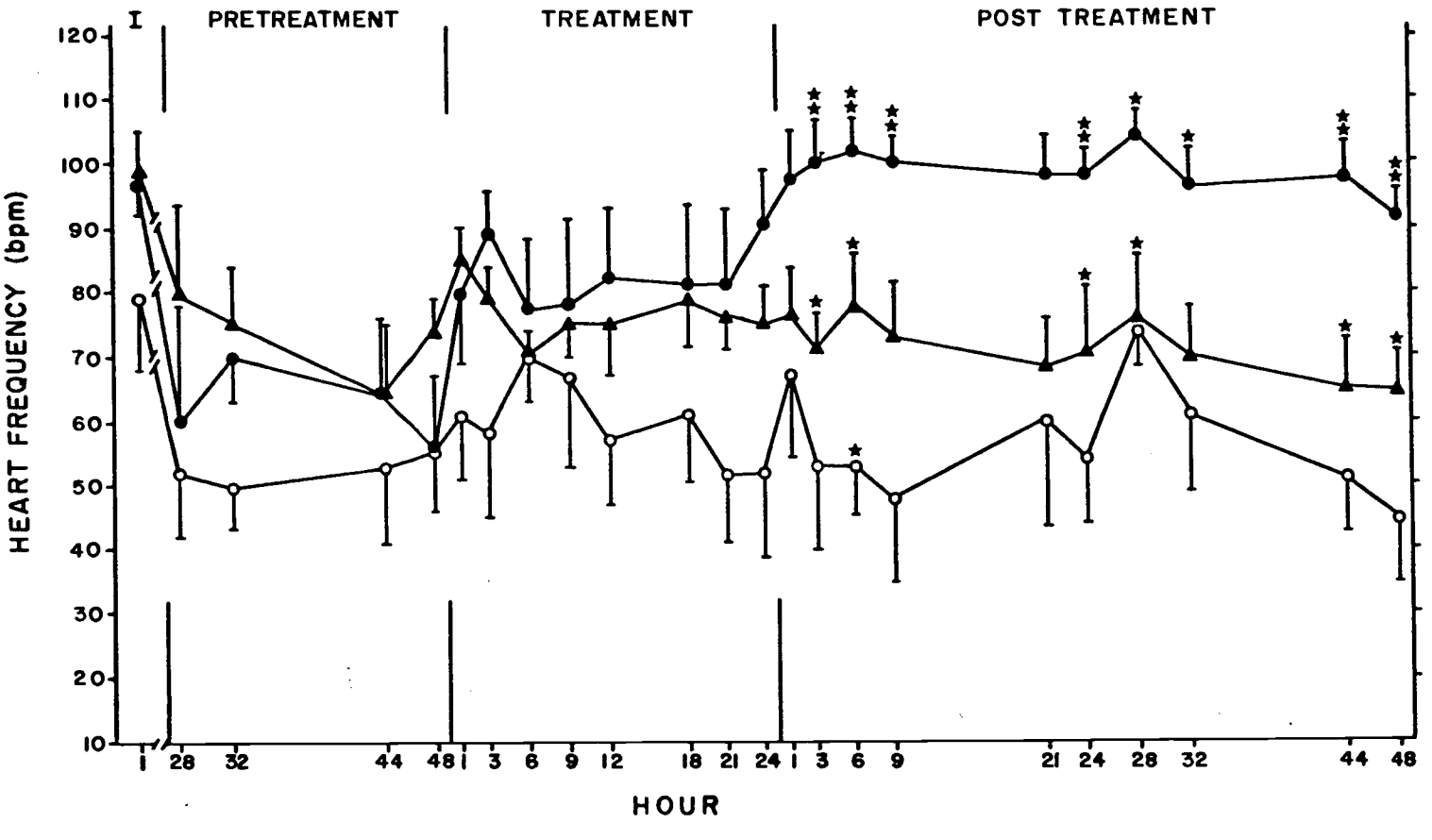


Figure 10. The mean heart rates of control (o), 1-naphthol- (▲) and carbaryl-treated (●) ghost shrimp from 28 hr pretreatment through 48 hr post treatment. Immediate post-implantation rates (I) are given for comparison. Bars represent ± 1 SEM. Stars above control curve represent comparison between control and 1-naphthol, those above 1-naphthol between 1-naphthol and carbaryl, and those above carbaryl between control and carbaryl. (* = $p < 0.05$; ** = $p < 0.01$).

Figure 11. Portions of the impedence heart recording from one control (a), one 1-naphthol- (b), and one carbaryl-treated (c) individual at 1 hr post-implantation (IM), 48 hr pretreatment, 3 and 18 hr treatment, and 24 and 48 hr post-treatment.

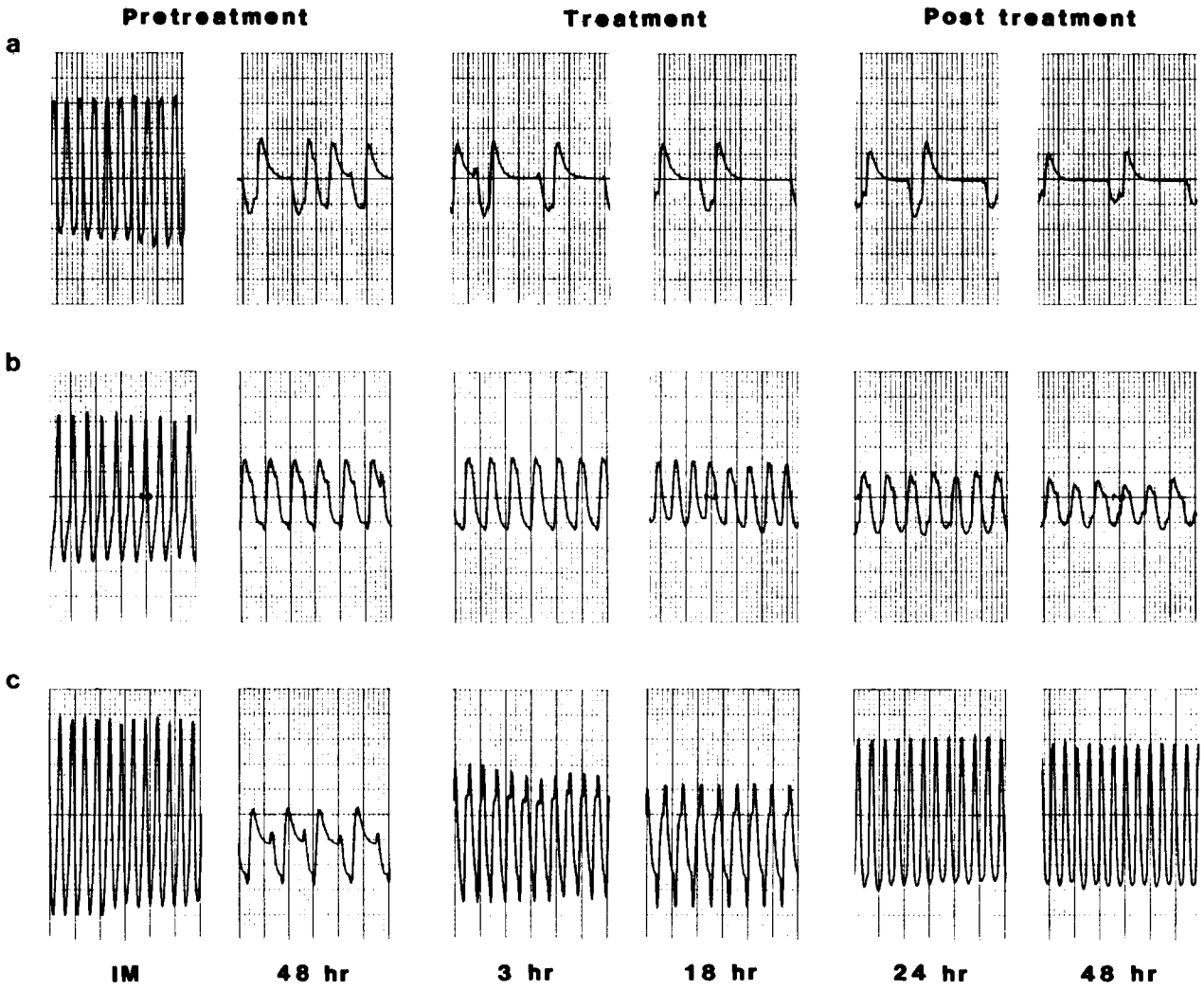

Pretreatment
Treatment
Post treatment
a
b
c
IM
48 hr
3 hr
18 hr
24 hr
48 hr

Figure 11.

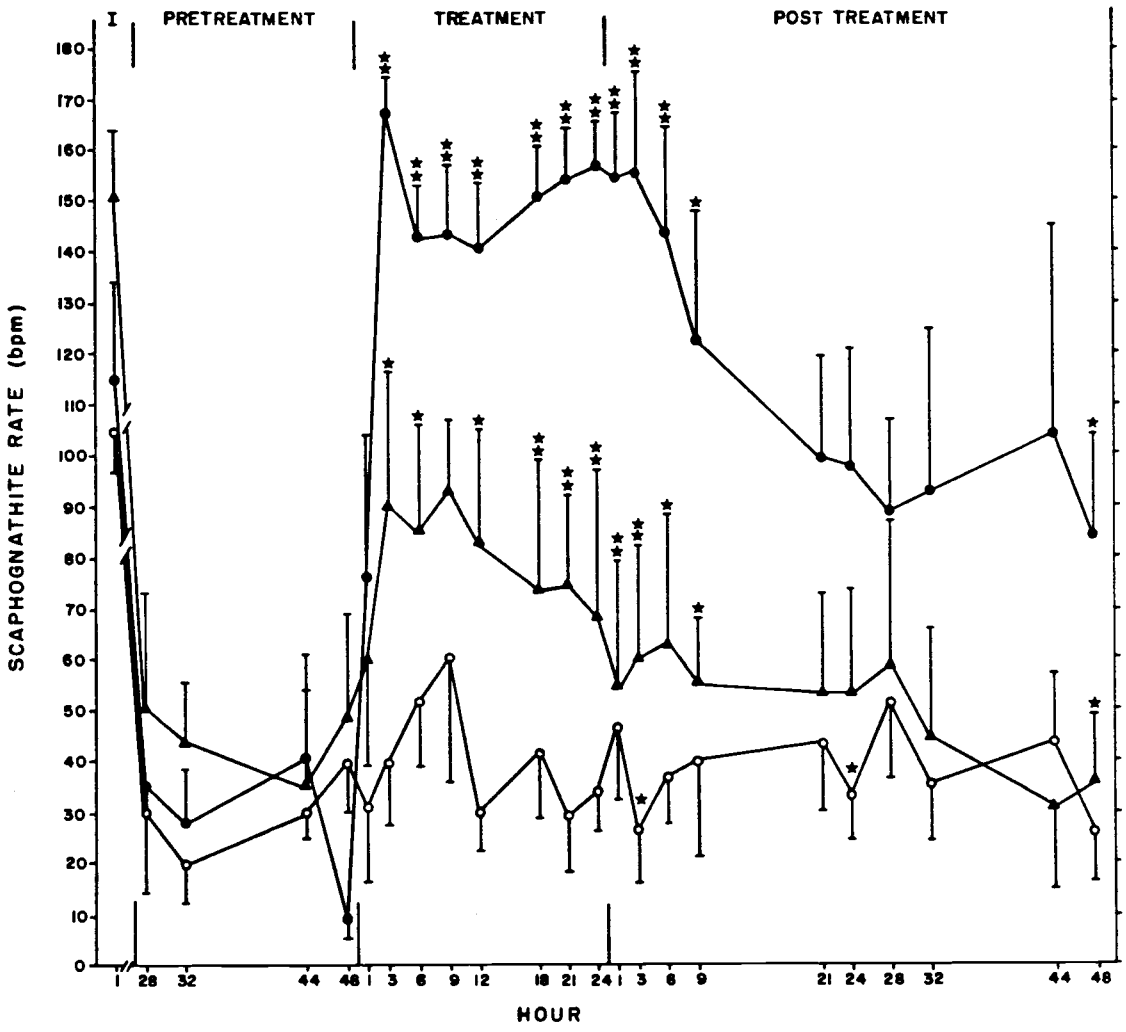


Figure 12. The mean scaphognathite frequencies of control (o), 1-naphthol- (▲), and carbaryl-treated (•) ghost shrimp from 28 hr pretreatment through 48 hr post treatment. Immediate post-implantation frequencies (I) are shown for comparison. Bars represent ± 1 SEM. Stars above control curve represent comparison between control and 1-naphthol, those above 1-naphthol between 1-naphthol and carbaryl, and those above carbaryl between control and carbaryl. (* = $p < 0.05$; ** = $p < 0.01$).

Figure 13. Portions of the impedance scaphognathite recording from a control individual (a), 1-naphthol- (b), and carbaryl-treated (c) at 1 hr post-implantation (IM), 48 hr pretreatment, 3 and 18 hr treatment, and 24 and 48 hr post-treatment.

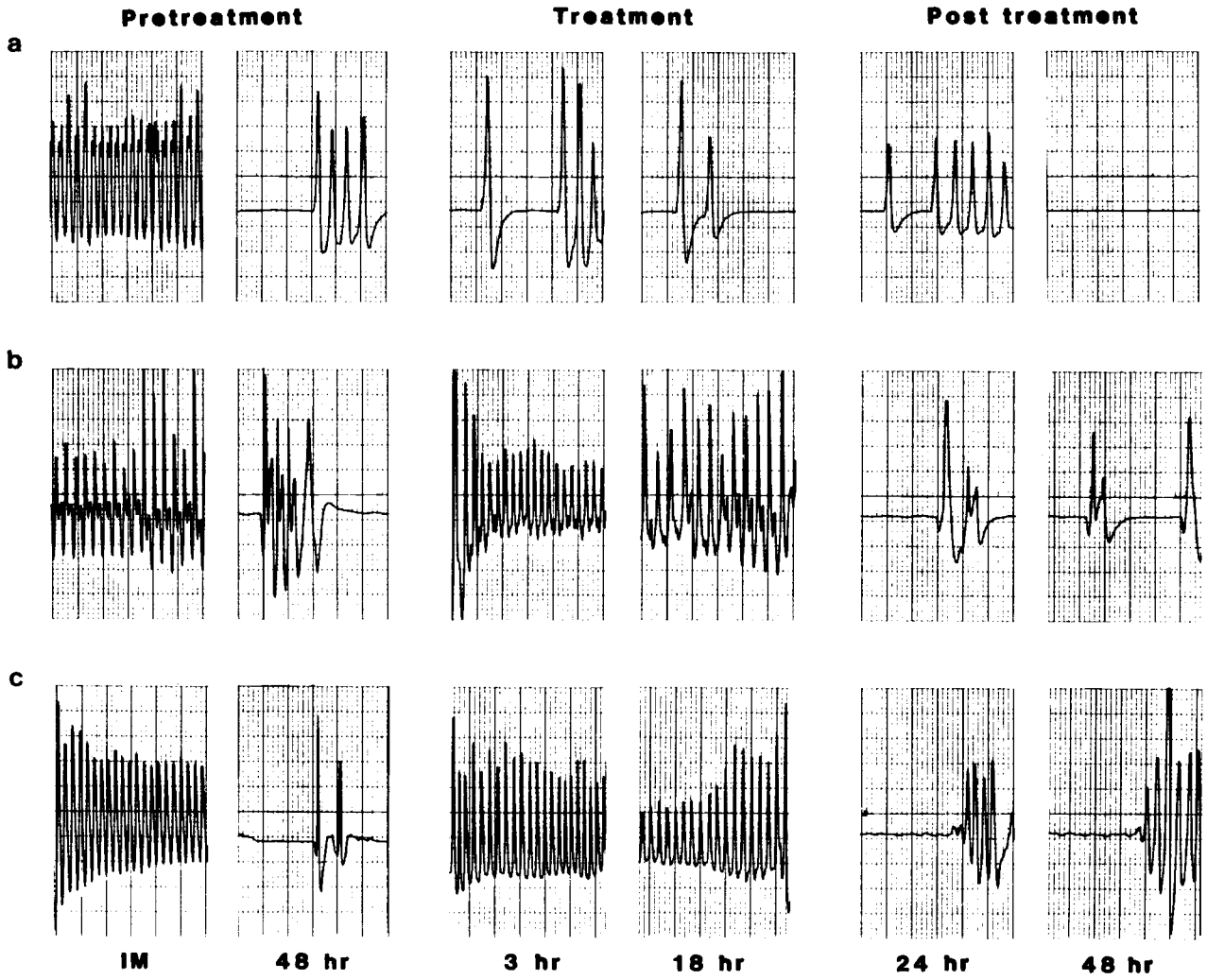

Pretreatment
Treatment
Post treatment
a
b
c
IM
48 hr
3 hr
18 hr
24 hr
48 hr

Figure 13.

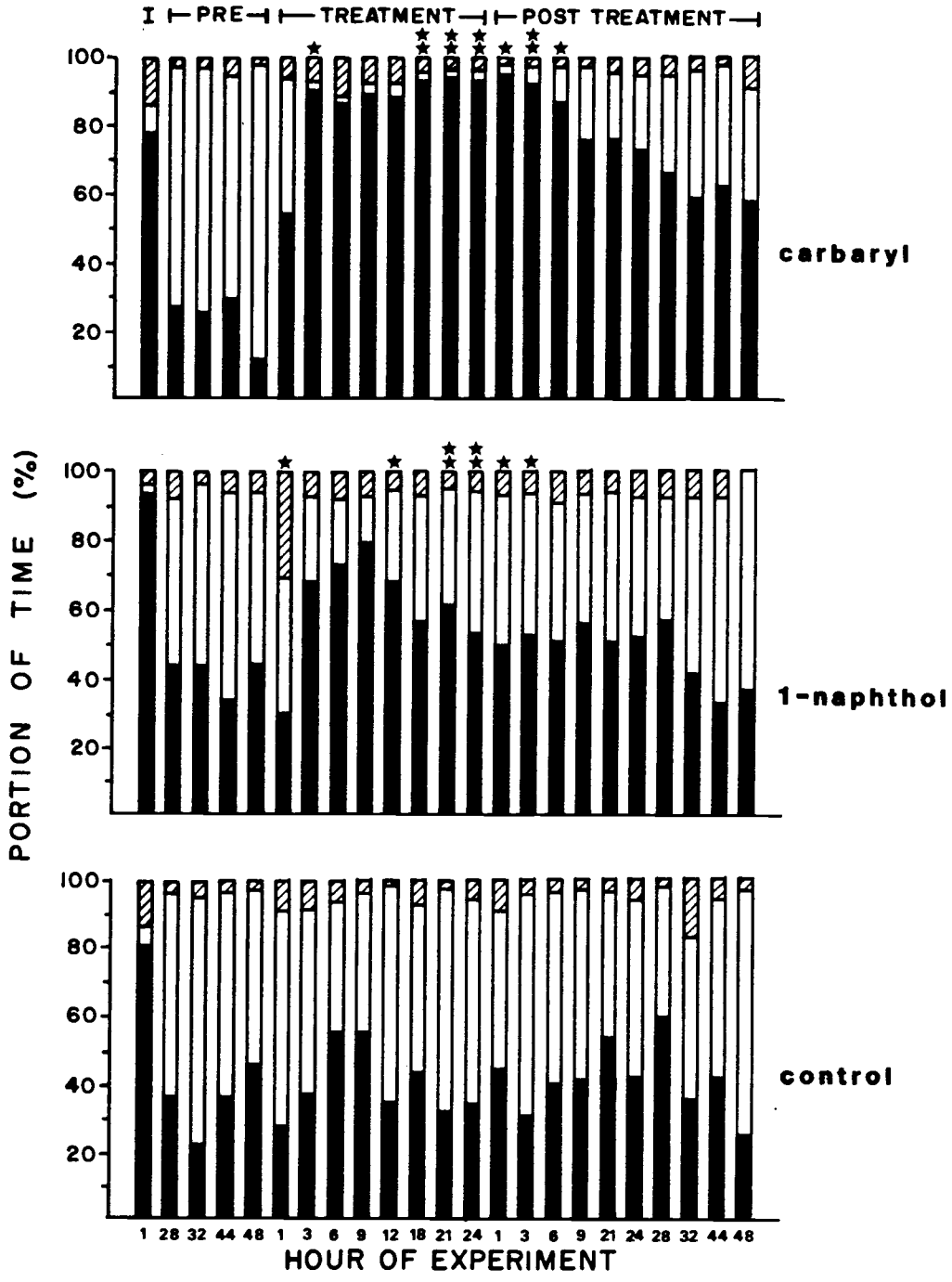


Figure 14. The percentage of time engaged in rhythmic beating (solid), arrhythmic beating (striped) and apnea (open) for control, 1-naphthol-, and carbaryl-exposed ghost shrimp from 28 hr pretreatment to 48 hr post treatment. One hour post-implantation patterns (I) are shown for comparison. Stars above control bars represent comparison between control and 1-naphthol, those above 1-naphthol between 1-naphthol and carbaryl, and those above carbaryl between control and carbaryl. (* = $p < 0.05$; ** = $p < 0.01$).

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CHAPTER 3

The release of mucosubstances by the ghost shrimp, Callianassa californiensis, exposed to carbaryl and 1-naphthol

INTRODUCTION

Carpenter (1927, 1930) reported that fish exposed to heavy metals quickly became covered with a film of mucus that was particularly thick over the gill epithelium and that the opercular movements of the fish seemed to indicate they were undergoing respiratory distress. Since then, other workers have noted increased mucus production accompanied by changes or damage in gill epithelia in fish exposed to a variety of toxicants including phenol (Mitrovic et al., 1968), acid (Ultsch and Gros, 1979; reviewed by Fromm, 1980), and ammonia (Arillo et al., 1979; Smart, 1976). This phenomenon has scarcely been studied in other aquatic organisms. Increased mucus secretion and severe damage to the epithelial tissue of the gills (ctenidia) and siphons occurred in the bent-nosed clam, Macoma nasuta exposed to the insecticide carbaryl (Armstrong, 1974). Mucus secretion has not been documented in crustaceans subjected to carbaryl or other pollutants.

Mucous secretions of vertebrates, including fish, generally contain glycoproteins with sialic acid residues. In invertebrates, sulfated acid mucopolysaccharides of proteoglycans seem to be the more important functional constituents of mucous secretions, although glycoproteins may be present also (Hunt, 1970).

Mucosubstances, whether glycoproteins or proteoglycans, are often secreted as a lubricant or as a coating to protect epithelia from abrasion, harsh chemicals or pathogens. The advantage of a mucus coating might seem minimal in crustaceans which have a chitinous exoskeleton, yet crustaceans possess epidermal tegumental glands which produce secretions containing mucopolysaccharides. These glands are found throughout the integument as well as in the fore and hind gut of most groups of crustaceans (Yonge, 1932). The functions of these glands and their secretions is not yet fully understood.

The purpose of these experiments was to determine whether ghost shrimp secrete mucosubstances when exposed to sublethal levels of carbaryl and 1-naphthol. Several observations prompted this study. First, it was noted that the test medium surrounding stressed ghost shrimp (see Chapter 2) became foamy within 9 hr after addition of carbaryl or 1-naphthol, the effect being more pronounced with carbaryl than with 1-naphthol. Secondly, the test medium seemed slightly cloudy or tinted and often clogged the extraction columns used to concentrate the toxicant. Finally, the changes noted above did not occur in toxicant solutions alone nor in solutions containing untreated ghost shrimp.

MATERIALS AND METHODS

Ghost shrimp weighing 6.6-17.3 g ($\bar{x}$ = 14.0 g) were placed in individual 700 ml containers of continuously aerated seawater which

was maintained at 12°C in a water bath. Shrimp remained in the containers for 24 hr, after which seawater samples were removed and frozen for later analysis. These samples were taken so that each animal could serve as its own control. The seawater was then completely removed from each container by low-pressure suction and was replaced with well-aerated, 12°C seawater containing either carbaryl or 1-naphthol at the 24 hr EC_{100} level-- initial levels of carbaryl and 1-naphthol were 0.194 and 8.22 mg/l, respectively. The ghost shrimp remained in the toxicant solutions for an additional 24 hr after which a second water sample was removed and frozen. The behavior of each ghost shrimp was noted at this time.

Seawater samples were analyzed for mucosubstances using a colorimetric assay developed by Whiteman (1973) and modified by Hall et al. (1980). This method, briefly outlined below, makes use of the interaction of the cationic dye Alcian Blue with the polyanionic carbohydrate residues of many proteoglycans and glycoproteins. Seawater samples were equilibrated with the Alcian Blue (Eastman, Rochester, NY) reagent for 2 hr to allow for complete formation of Alcian Blue complexes. The samples were then centrifuged at 3000 xg for 30 min and the supernatant aspirated and discarded. The pellet was washed twice with an EtOH/buffer solution and centrifuged in between each wash. The two EtOH washes were designed to remove any unbound Alcian Blue from the sample. Sodium diisobutyl sulfosuccinate solution (Pfaltz and Bauer, Inc., Waterbury. CT) was added to the pellet to dissociate the dye from the complexes. Samples were sonicated for 20 sec at half power with a Kontes

ultrasonicator (Vineland, NJ). The absorbance of the resulting clear blue solution was read at 620 nm in a Varian Techtron UV-Vis Spectrophotometer (Model 635, Walnut Creek, CA).

A chondroitin 6-sulfate (Sigma Chemical Co., St. Louis, MO) standard curve was linear over the range of 0-8 μg (Figure 15). Chondroitin 6-sulfate was chosen as a standard because its binding with Alcian Blue has been shown to be linear (Whiteman, 1973) and because chondroitin sulfate has been found in the tissues of various crabs and shrimp (Ehrlich et al., 1981; Nader et al., 1983; Cassaro and Dietrich, 1977). Chondroitin 6-sulfate contains 2 anionic groups per repeating D-glucuronate-N-acetyl-D-galactosamine unit and binds one Alcian Blue molecule under these experimental conditions (Whiteman, 1973). The standard curve was used only as an index of the linear nature of Alcian Blue binding as the exact composition of crustacean secretions is unknown. Absorbance units, therefore, represent the relative amounts of mucosubstance secreted by the ghost shrimp during the various treatments.

Data was analyzed with a one-way analysis of variance (ANOVA) and Fisher's least significant difference (LSD) test.

RESULTS

During the control period ghost shrimp showed no signs of unusual behavior, nor did the medium become cloudy or foamy. When exposed to the 24 hr EC_{100} of carbaryl or 1-naphthol the same ghost shrimp developed Stage 3 or Stage 4 behavior (see Chapter 1)

and the water became cloudy and foamy, usually within 9 hr after exposure.

Figure 16 shows that during the control period neither group of ghost shrimp produced measurable mucous secretions, since the absorbance of the seawater samples (animal controls) were not significantly different from the seawater blank used in the assay. Following exposure to carbaryl or 1-naphthol for 24 hr, there was a 3 to 4 fold increase in the absorbance of the seawater samples, indicating a significant ($p<0.01$) increase in relative mucus production. The increase in the absorbance was not simply caused by the addition of the toxicants since toxicant solutions (toxicant controls) alone did not have an increased absorbance. In fact, the toxicant controls had significantly lower ($p<0.05$) values than the paired animal controls. The reason for this is not clear due to the relatively small sample size. Ghost shrimp exposed to carbaryl produced more secretions than did ghost shrimp exposed to 1-naphthol ($p<0.05$) which agrees with earlier observations of the overall appearance of the water during the two different treatments.

DISCUSSION

Hypersecretion of mucus, which occurs soon after fish are exposed to pollutants, may be a protective mechanism shielding epithelial tissues from pollutants and slowing the inflammatory response which may directly damage the tissues. Arillo et al. (1979) reported an increase in mucus production in fish exposed to

low levels of ammonia long before any histological changes were noted in the gill epithelia. In another study mucus-covered gills became swollen, the epithelium became thickened, and hemorrhage was observed in fish chronically exposed to ammonia (Smart, 1976). Both Brown et al. (1968) and Mitrovic et al. (1968) reported mucus production, and lifting and sloughing of the gill epithelium in response to zinc and detergent, and phenol, respectively. Murray and Fletcher (1976) suggested that stress responses may initiate mucus production since they noted explosive release of mucus in fish that were exposed to stressful conditions (enforced activity). Skidmore and Tovell (1972), observed a lifting of the epithelial layer from the basal lamina and the edema of the resulting extracellular space in response to zinc sulfate. They suggested that this damage was part of a typical acute inflammatory reaction of tissues in contact with a pollutant.

My experiments have shown that glycoproteins containing acidic residues or acid mucopolysaccharides are released into the water when ghost shrimp are exposed to carbaryl or 1-naphthol. The source of the material and its function cannot be determined from this study. Mucus release by crustaceans has rarely been reported even as part of the normal behavior of the animals. Cahoon (1982) observed the secretion of a mucosubstance by the raptorial calanoid copepod Euchirella venusta which aided it in snaring prey. The peritrophic membrane surrounding the fecal pellets of many decapods may be composed of mucus (Georgi, 1969). Thompson (1972) gave morphological and histochemical evidence that the thalassinid shrimp

Upogebia pugettensis makes use of mucous secretions in forming the lining of its permanent burrows. Specialized tegumental glands in the hindgut were reported to be the source of the secretions described in these studies. In addition, both Dall (1965) and Doughtie and Rao (1981, 1982) found abundant tegumental glands in the pleural region of the shrimp that they studied.

The nature of the secretions of the crustacean tegumental glands and their functions has long been debated. Farkas (1927) described the secretions of the glands as mucus-like, based on histological staining. Yonge (1932) claimed that the glands produced the new cuticle during ecdysis. He observed that the glands were found throughout the integument, that their secretory patterns seemed to follow the molting process and that carbohydrate stains could stain components of chitin as well as mucus. Dall (1965) reported that the epicuticle of the shrimp Metapenaeus sp. stained only faintly with Alcian Blue and that the procuticle did not stain at all. The tegument glands, however, stained intensely with Alcian Blue. This would tend to support the role of tegumental glands in mucus production, not cuticle formation.

Although the protective role of mucus in fish exposed to toxicants seems to be well established, there are no other studies which might support a similar role in crustaceans. If the mucus is not being secreted in response to irritation of epidermal tissue, how might carbaryl and 1-naphthol induced release of mucus be explained? The neurotoxic action of both of these compounds may stimulate secretory activity. Doughtie and Rao (1982) reported the

direct innervation of Type B rosette glands in the gills of Palaemonetes pugio, but other reports of innervation of tegumental glands are few (see Foster and Howse, 1978). It is more likely that the secretion is hormonally controlled, especially since the normal secretory pattern seems to follow the molt cycle (Yonge, 1932; Doughtie and Rao, 1982) which is controlled by hormones. Insecticide-induced release of cuticular plasticization factor was seen in Rhodnius prolixus dosed topically with the methylcarbamate Zectran (Maddrell and Reynolds, 1972). Therefore insecticides which cause hyperactivity of the nervous system could cause hypersecretion of neurohormones which may control mucus secretion.

Even if mucus release is principally caused by the neurotoxic nature of carbaryl and 1-naphthol, gill tissue might still be affected. The chitin layer covering the gills is very thin and the cuticle is an important route of entry of insecticides into insects and probably crustaceans as well. Gill tissue may therefore be the first to come in direct contact with insecticides. Inflammatory reactions similar to those seen in fish may damage gill tissue. Indirectly, secretion of mucosubstances into the water may interfere with oxygen uptake no matter what the source of the material. Mucosubstances drawn into the branchial cavity by respiratory currents might deposit on the gills and interfere with oxygen consumption. Ultsch and Gros (1979), for example, found that the diffusivity of oxygen in fish mucus was only about 70% that in water. A mucus coating could also interfere with oxygen consumption by changing the ventilatory patterns. Activity designed to clear

gills of mucus might not lead to optimum oxygen uptake. To address this question, measurements of oxygen consumption in carbaryl and 1-naphthol exposed ghost shrimp were made and reported in Chapter 4.

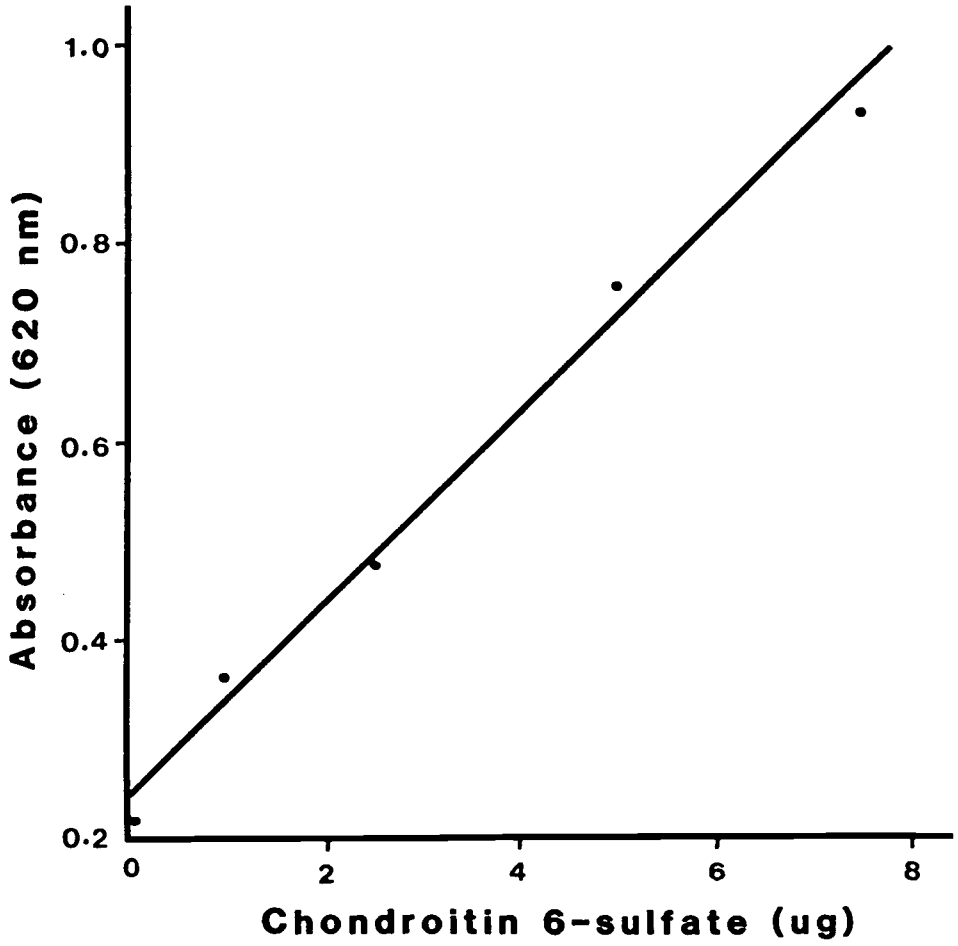

Figure 15. Quantitative interaction between chondroitin 6-sulfate and Alcian Blue dye.

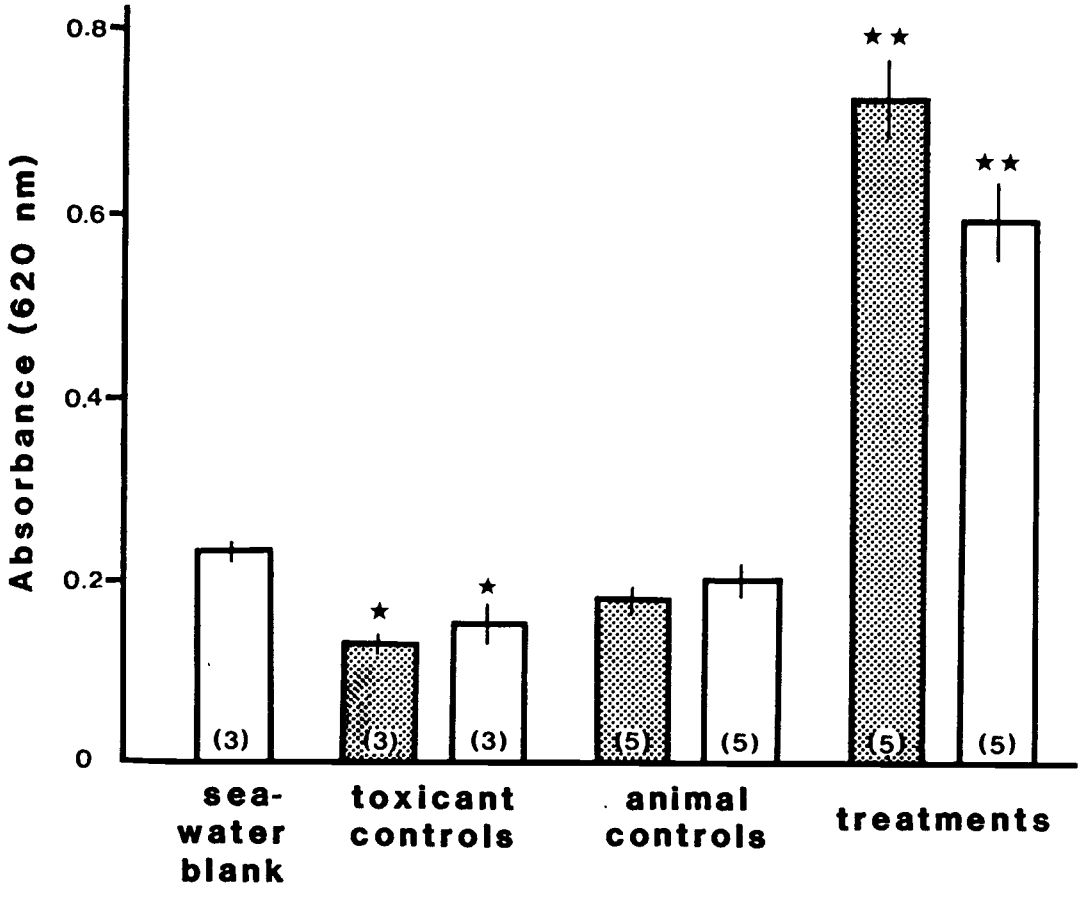

Figure 16. The relative amount of Alcian Blue-acid mucopolysaccharide complexes in seawater under various experimental conditions. Bars pertaining to carbaryl are stippled, those referring to 1-naphthol are open. Seawater blanks and toxicant controls were prepared identically except that the latter contained either carbaryl or 1-naphthol. Animal controls were samples taken from containers which held shrimp in untreated seawater for 24 hr. Treatments were samples taken from containers which held shrimp exposed to the toxicants for 24 hr. Bars represent ± 1 SEM. Number of individuals (or samples) is in parentheses. (* = $p < 0.05$; ** = $p < 0.01$).

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CHAPTER 4

The effects of carbaryl and 1-naphthol on the metabolic rate of the ghost shrimp, Callianassa californiensis

INTRODUCTION

Metabolic rates, measured as oxygen consumption rates, have been used to detect overall physiological changes associated with both natural and pollution stresses (see review by Vernberg, 1983). Presumably pollutants can also act upon exposed respiratory membranes to affect oxygen uptake, regardless of any metabolic effects they might have. Few studies have assessed the impact of insecticides on oxygen consumption in crustaceans (Andryuschencho, 1972; Rao et al., 1982, 1979; McKenney, 1982).

The purpose of this study was to determine the effect of the carbamate insecticide carbaryl and its primary daughter product 1-naphthol on the oxygen consumption rate of the thalassinid ghost shrimp Callianassa californiensis. An increase in scaphognathite frequency has been seen in ghost shrimp exposed to carbaryl and 1-naphthol (Chapter 2). However secretion of mucosubstances by treated ghost shrimp (Chapter 3) could interfere with their ability to obtain oxygen across the gill epithelium. Normally ghost shrimp have low oxygen consumption rates and are able to regulate their metabolic rate down to very low oxygen tensions (Thompson and Pritchard, 1969), which is adaptive for survival in a hypoxic environment. Alteration of the metabolic rate of these ghost shrimp

by pollutants could affect their ability to cope with an oxygen-limited environment.

MATERIALS AND METHODS

Oxygen consumption rates were measured using a Gilson differential volume respirometer (Model GR-14, Gilson Medical Electronic, Inc., Middleton, WI). Male and non-gravid female ghost shrimp 3.0-15.8 g ($\bar{x}$ = 7.6 g) were used in this study. No weight- or sex-specific trends were noted (see appendix Tables 5 and 6) so data from all individuals were pooled.

Ghost shrimp were placed individually into 250 ml Erlenmeyer flasks containing 75 ml of test medium. This volume of medium was chosen as it provided complete coverage of the gills of even the largest individuals, while minimizing the volume that would have to be equilibrated in the respirometer. The respirometers included a vial which contained 1 M KOH and filter paper wicks, which acted as a CO_2 trap. The test medium for control animals was well-aerated (6.0 ml/l O_2), filtered (0.45 μ), 12°C seawater. For experimental animals carbaryl or 1-naphthol was added to the seawater. Initial levels of 0.203 ± 0.005 mg/l of carbaryl and 15.7 ± 0.1 mg/l of 1-naphthol resulted in the loss of equilibrium of all of the test animals within the 4.5 hr dose period (4.5 hr EC_{100}; toxicant concentrations declined approximately 70% over the course of the exposure). These levels were higher than those used in the previous studies (Chapter 1, 2 and 3) due to the lower volume of

test media used and the shorter exposure period in this study. As the volume of media decreases, so does the total amount of toxicant to which the animal is exposed, so the concentrations used previously did not produce the desired behavioral end point in this study.

Five active flasks, four experimental and one blank containing only test media, were used in each experiment. Oxygen fluctuations in the blank, usually minimal, were used to correct the readings of the experimental flasks. A 1200 ml cylinder containing water-saturated air served as reference flask and was approximately equal to the total volume of the active flasks. Animals were equilibrated in the experimental flasks for 2 hr prior to measuring oxygen consumption. The equilibration period ensured equal temperature and pressure throughout the system, allowed shrimp to recover from handling, and allowed them to be exposed to toxicant for 2 hr prior to measurements. Previous experiments (Chapter 2) indicated that the greatest increase in scaphognathite rate occurred by approximately 3 hours after exposure to toxicants. Oxygen consumption rates were therefore begun at 2 hr into the exposure period and were terminated at 4.5 hr.

After the equilibration period, the system was closed and readings of oxygen uptake were made at 15 min intervals for 90 min or until the capacity of the micrometers had been reached. After the system was reset, measurements were taken for a second interval of only 60 min because oxygen consumption usually exceeded the range of the micrometers after only 1 hr at this stage of the exposure.

Oxygen consumption was therefore measured for 2.5 hr during the 4.5 hr exposure period. At the end of this period, the behavior of each animal was described and the final concentration of toxicants was determined to be 0.059 ± 0.002 mg/l for carbaryl and 4.5 ± 0.2 mg/l for 1-naphthol. Volume changes were converted to µl O_2/ g wet wt/ hr at STP using half hour intervals and oxygen consumption rates were reported for 2.5, 3.0, 3.5, and 4.0 hrs. after treatment. Data was analyzed using a one-way analysis of variance (ANOVA) and Fisher's Least Significant Difference (LSD) test.

RESULTS

Oxygen consumption rates are shown in Figure 17. The oxygen consumption rates of control ghost shrimp remained constant over the period with a mean rate of 21.9 ± 0.9 µl O_2/g/hr. This compares favorably with the mean rate of 19.8 ± 2.3 µl O_2/g/hr obtained by Eddy (unpublished data) on unstressed male and female ghost shrimp (2.0-13.7 g) at 12°C using a Gilson differential volume respirometer. Ghost shrimp exposed to the toxicants displayed metabolic rates significantly higher than controls ($p<0.01$) even after 2.5 hr. Initially, the rates of the 1-naphthol-exposed ghost shrimp were significantly lower ($p<0.01$) than that of carbaryl-exposed ghost shrimp. By 3.5 hr, the oxygen consumption rates of the 1-naphthol-treated animals had increased to the level of the carbaryl-treated animals. The metabolic rates of the carbaryl-treated ghost shrimp did not change significantly, but the

rates of the 1-naphthol-exposed ghost shrimp increased throughout the measurement period.

By the end of the 4.5 hr exposure period, all but one of the 1-naphthol-exposed and half of the carbaryl-exposed ghost shrimp were in Stage 3 of the intoxication response (loss of upright posture, constant muscle spasms; see Chapter 1). Only one of the 1-naphthol-treated and the other half of the carbaryl-treated ghost shrimp had reached Stage 4 intoxication (loss of upright posture, partial paralysis; see Chapter 1). The metabolic rates of the carbaryl-exposed Stage 3 ghost shrimp were generally higher (but not significantly) than the carbaryl-exposed Stage 4 ghost shrimp ($\bar{x}$ = 60.1 $\pm$ 5.6 and 45.1 $\pm$ 4.7 µl O_2/g/hr, respectively).

DISCUSSION

Many factors are known to influence the metabolic rates of crustaceans (see review by Vernberg, 1983). These factors include size of the individual, temperature, salinity, light, oxygen level, activity, digestive state, and circadian rhythms. In the present study, precautions were taken to ensure as much uniformity as possible in the animals selected and in the experimental conditions.

One criticism of the differential volume respirometry method is the possible depletion of oxygen in the aqueous medium which may affect the metabolic rate of the animals (McMahon and Wilkens, 1983). This is partially circumvented by the high surface-to-volume ratio of water to air which allows for free exchange of oxygen

across the water-air interface. However, even if the oxygen content of the water were to decrease slightly over the course of 4.5 hr, Thompson and Pritchard (1969) found that the metabolic rate of ghost shrimp becomes dependent upon oxygen content only below a concentration of 0.8 ml/l. These animals, therefore, must be able to tolerate hypoxia. The stability of the control rates suggests that any decrease in oxygen level which may have occurred during the test period did not affect oxygen consumption rates.

Activity levels of the ghost shrimp changed upon exposure to carbaryl and 1-naphthol. Control ghost shrimp did maintain some minimal amount of activity, so oxygen consumption values reported are "routine" metabolic rates rather than resting rates. During Stage 3 intoxication ghost shrimp were very active, displaying constant convulsions. Two to 3.5 fold increases in oxygen consumption have been seen in several crustaceans even after 10 min of activity (Herreid et al., 1983; Rutledge and Pritchard, 1981). Jarvis (1973) reported a 2.0 to 2.5 fold increase in oxygen consumption in ghost shrimp subjected to forced activity. In the present study, a 2.3 fold increase in oxygen consumption was seen during toxicant treatment compared with controls. If this increase is entirely attributable to toxicant-induced increases in muscle activity, it is hard to explain why paralyzed Stage 4 ghost shrimp displayed rates similar to convulsive Stage 3 ghost shrimp. During Stage 4 intoxication ghost shrimp did not appear active, thus, increases in activity alone could not account for the increase in oxygen consumption during intoxication. Heslop and Ray (1959)

examined the oxygen consumption of physically-stressed and DDT-dosed cockroaches. Individuals undergoing either treatment initially displayed increased activity. All of the DDT-treated roaches and some of the physically-stressed roaches eventually became paralyzed. All of the roaches which became paralyzed displayed a surge in oxygen consumption just before paralysis set in. However, those physically-stress animals which never became paralyzed showed no surge in oxygen consumption rate even though they actively struggled. The investigators felt that both the increase in metabolic rate and the paralysis were due to stress-induced release of neurohormones.

The metabolism of the toxicants themselves may account for some of the increase in the metabolic rate. It is known, for example, that two enzymes involved in the metabolism of carbaryl in cockroaches and locusts are oxygen-dependent (Cocks, 1975). Cytochrome P-450 mediated mixed function oxidases (MFO) metabolize foreign compounds, and are present in marine crustaceans as well as in insects (Lee, 1981). Metabolism of carbaryl and perhaps 1-naphthol by the MFO system could increase the oxygen demand of the ghost shrimp.

Few studies have examined the effects of pesticides on the metabolic rates of crustaceans. Andryuschenko (1972) tested the effects of DDT on the oxygen consumption of the Black Sea shrimp Palaemon squilla and reported reductions in oxygen consumption of 30 and 40% at 10^{-2} and 10^{-3} mg/l, respectively. Sodium pentachlorophenol, a broad-spectrum biocide found in many commercial

pesticides, inhibited oxygen consumption in blue crab Callinectes sapidus isolated gill, muscle, and hepatopancreas tissue (Rao et al., 1979). Gill damage as well as decreased oxygen consumption were seen in grass shrimp Palaemonetes pugio exposed to dithiocarbamates which are commonly used as herbicides, fungicides, and nematocides (Rao et al., 1982). However, the estuarine mysid Mysidopsis bahia displayed elevated oxygen consumption rates when chronically exposed to sublethal levels of the chlorinated pesticide Endrin (McKenney, 1982).

Neurotoxic insecticides have been shown to increase oxygen consumption in insects at levels which increase spastic muscle activity, and to decrease it at higher levels where cellular damage actually occurs (Keister and Buck, 1974). This may explain why a single pesticide can increase oxygen consumption in one case and decrease it in another. Skidmore (1970) found that oxygen consumption increased in fish that struggled when initially placed in a zinc sulfate solution, but then declined rapidly once zinc-induced damage to gill epithelia had occurred. There have been examples of carbaryl-induced tissue damage in both marine bivalves and in insects. Armstrong (1974) reported necrosis of the epithelial tissue of the gills, mantle and siphon in the bent-nosed clam Macoma nasuta exposed to carbaryl. The most severe damage was seen in the gills and the damage was dose-dependent. Carbaryl caused distinct histological changes in the epithelial cells of the alimentary canal and within the nervous system of the Red cotton bug Dysdercus koenigi when it was applied topically (Sharma, 1968).

Extensive tissue necrosis has also been observed in larval Earias insulana, a lepidopteran, exposed to carbaryl for 24 hr (Hassanein et al., 1968). It might be inferred that at the levels tested in this study carbaryl and 1-naphthol increased locomotor and metabolic activity, but did not cause damage to tissues, especially to gill tissues, since oxygen consumption increased rather than decreased.

Oxygen consumption measurements are often hard to interpret when presented alone. For example, water soluble fractions (WSF) and oil-water dispersions (OWD) of No. 2 fuel oil at the LC_{50} level caused a decrease in oxygen consumption in grass shrimp Palaemonetes pugio, but caused an increase in the metabolic rate of opossum shrimp Mysidopsis almyra (Neff et al., 1976). Likewise, petroleum effluents caused a decrease in oxygen consumption in Penaeus aztecus, but an increase in oxygen consumption in Penaeus duorarum (Steed and Copeland, 1967). Thurberg et al. (1973) found that sublethal levels of cadmium (Cd) caused a decrease in the oxygen consumption of isolated gill tissue of both green crabs Carcinus maenas and rock crabs Carcinus irroratus, but Thurberg et al. (1977) found the opposite with gill tissue from the lobster Homarus americanus. An increase in oxygen consumption of isolated gills of juvenile blue crabs, Callinectes sapidus, exposed acutely to 1.0 ppm benzene was seen in a study by Cantelmo et al. (1982). However, a decrease was seen in gills excised from crabs that had been exposed chronically to the same concentration. Intact crabs exposed chronically showed a decrease in oxygen consumption as well.

It is difficult to compare the respiratory effects of one

pollutant with another or to compare the results of one experiment with another because there is little standardization among studies. Not only do experimental conditions differ, but the species tested and the developmental stages used are often different. In addition, responses can change with the level of toxicant used and with the duration of the exposure. Comparisons between whole animals and isolated tissues are even more difficult because behavioral components cannot be taken into account when dealing with isolated tissues. Although Bayne et al. (1985) stressed the importance of combining oxygen consumption measurements with behavioral observations, few researchers discuss toxicant-induced behavioral changes when interpreting oxygen consumption data, especially at low levels of toxicants.

In conclusion both carbaryl and 1-naphthol increased the oxygen consumption rates of exposed ghost shrimp over controls at 4.5 hr EC_{100} levels. Carbaryl-exposed ghost shrimp achieved maximum rates by 2.5 hr and maintained these rates until the end of the measurement period. The rates of the 1-naphthol-exposed ghost shrimp increased throughout the experimental period and achieved rates similar to carbaryl-treated ghost shrimp by 3.5 hr exposure. The increased metabolic rates may be due, in part, to the increased muscle activity of the treated ghost shrimp, though paralyzed and convulsive individuals had similar oxygen consumption rates. Metabolism of the toxicants themselves may also add to the increase in overall metabolic rate. The increased oxygen consumption rates seem to rule out damage to gill or other tissue as a result of

exposure to the toxicants at these levels. However, because both carbaryl and 1-naphthol significantly increase the metabolic rates of ghost shrimp, this might impair their ability to survive in an oxygen-limited environment.

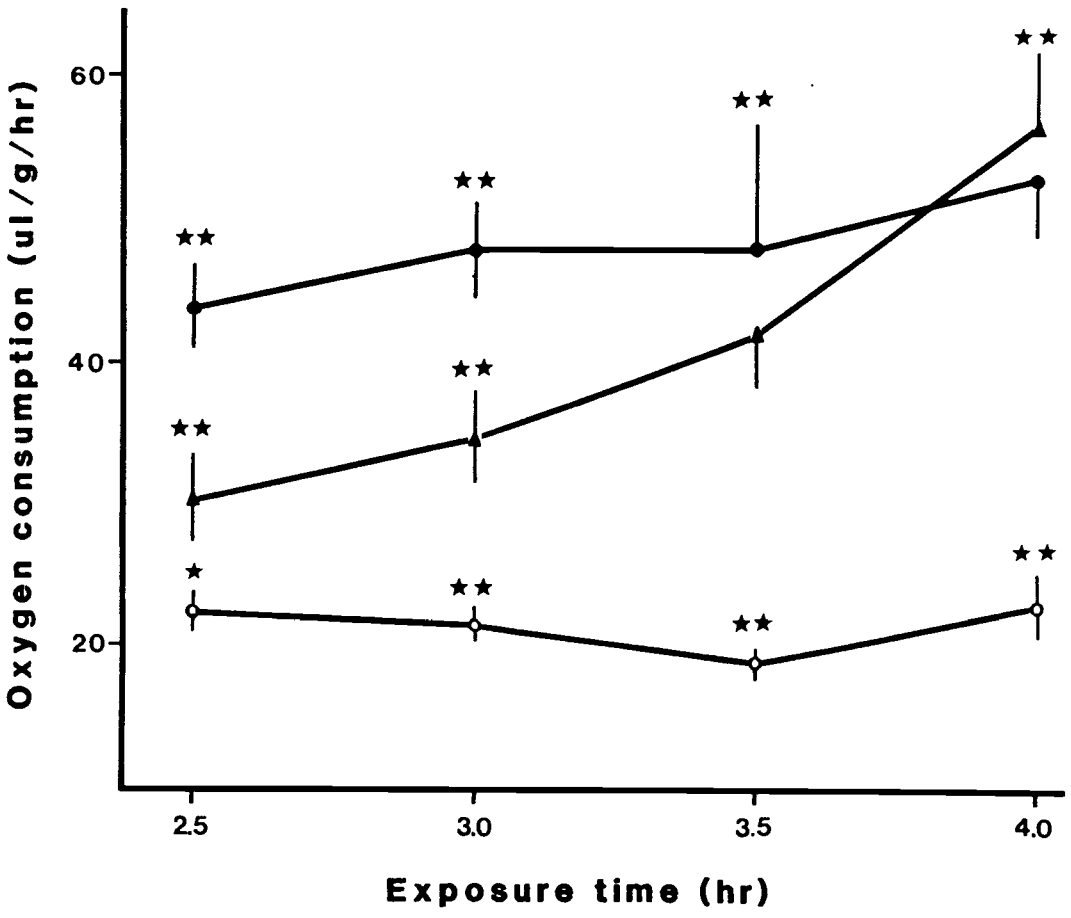

Figure 17. The effect of carbaryl and 1-naphthol on the oxygen consumption rates of ghost shrimp. Data points are means of rates taken over a 30 min period. Bars represent ± 1 SEM. Stars above control curve (○) represent comparison between control and 1-naphthol, those above 1-naphthol (▲) between 1-naphthol and carbaryl, and those above carbaryl (●) between control and carbaryl. (* = $p < 0.05$; ** = $p < 0.01$).

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SUMMARY AND CONCLUSIONS

In this series of investigations carbaryl and 1-naphthol treated ghost shrimp displayed hyperactivity and convulsions followed by paralysis. These behavioral changes are common in insects and crustaceans exposed to neurotoxic insecticides (Keister and Buck, 1974; Kuhr and Dorough, 1976; Naqui and Ferguson, 1970; Armstrong, 1974). During acute intoxication oxygen consumption was high in both groups of treated ghost shrimp, but only carbaryl-treated ghost shrimp showed significant increases in scaphognathite rates. Neither of the two groups displayed heart rates significantly different from controls during treatment. Increased mucus secretion was noted during toxicant exposure, especially during exposure to carbaryl. Recovery from 1-naphthol treatment was rapid once toxicant solutions were removed, but recovery from carbaryl intoxication was slower. Scaphognathite rates decreased during the recovery period, but were significantly higher than pretreatment levels at the end of 48 hr. Heart rates of carbaryl-exposed ghost shrimp, however, increased during post treatment and were still elevated at the end of 48 hr.

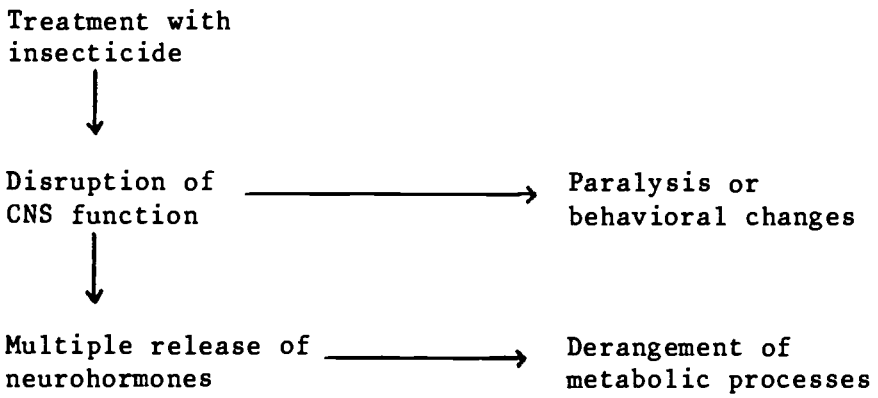
In general, heart rate may not be a good indication of cardiac performance; stroke volume and vascular resistence in the gills may be the major factors affecting cardiac output and perfusion (Wilkens, 1981; McMahon and Wilkens, 1983). Scaphognathites, which have a limited capacity for changes in stroke volume, have a greater range of beat frequencies (McMahon and Wilkens, 1983). Changes in

ventilation, therefore, may show up primarily as changes in scaphognathite frequency.

1-Naphthol produces similar behavioral changes and similar increases in oxygen consumption and mucus production as carbaryl. However, changes in heart and scaphognathite rates were minimal. 1-Naphthol may have a different mode of action than carbaryl. For example, DDT and carbaryl affect different components of the nervous system, but often produce similar changes in muscle activity and oxygen consumption in insects (Keister and Buck, 1974). 1-Naphthol and carbaryl may, in fact, have the same mode of action, but different rates of penetration or metabolism could modify the symptoms produced. Carbaryl has the ability to penetrate the insect cuticle and to cross cell membranes quite rapidly and therefore would reach its target rapidly to produce nervous system impairment (Ahmad et al., 1980; Kuhr and Dorough, 1976). 1-Naphthol with its polar hydroxyl group may not be able to cross the cuticle and cell membranes as easily as carbaryl, which may explain why it must be present at such high levels to produce symptoms of intoxication. Once 1-naphthol enters the animal there is evidence from studies with insects, that the hydroxyl group allows 1-naphthol to be rapidly conjugated or excreted as free 1-naphthol (Terriere et al., 1961). The cytochrome P-450 dependent mixed function oxidases (MFO) probably provide the main mechanism of metabolism of carbaryl in insects and crustaceans (Kuhr and Dorough, 1976; Lee, 1981). Metabolism produces polar metabolites that can be more easily excreted (Brattsten et al., 1986). However, some metabolites

produced in insects (Ahmad et al., 1980) have some residual ability to inhibit AchE (Mount and Oehme, 1981) and so metabolism may not always completely detoxify carbaryl. Different modes of action, different rates of penetration, and different mechanisms and rates of metabolism may explain differences in symptomology of carbaryl and 1-naphthol. Greater variability in response was noted during 1-naphthol treatment than with carbaryl treatment. This may contribute to the lack of significant increase in rates in the 1-naphthol treatment group.

The cause of death in carbamate poisoning is unclear. A remarkable degree of recovery from paralysis is often seen in insects treated with carbamates. These insecticides are reversible AchE inhibitors (Miller, 1976; Kuhr and Dorough, 1976), so the inhibition of AchE may not be the cause of death in carbamate poisoning. In this study paralyzed Stage 4 and Stage 5 ghost shrimp often recovered completely once placed in clean seawater. Alternate causes of death have therefore been proposed, one of which involves the mass release of neurohormones. The theory of Maddrell and Reynolds (1972) is presented below:



The individual's physiological condition before poisoning, environmental factors, and duration of exposure would determine whether the insecticide poisoning would be fatal.

In a field study (Karenin et al., 1967), carbaryl as Sevin 80S was applied to an Oregon mudflat (temp. 12.5°C) at 10 lb/acre and carbaryl and 1-naphthol levels in the sediment were monitored for 6 weeks. Two hr after application the carbaryl level was 5.4 ppm and the 1-naphthol level was 5.3 ppm in the top 1 in. of sediment. These levels correspond to over 30 times the EC_{100} for carbaryl and to the EC_{91} for 1-naphthol as determined in this study. After 24 hr (and 2 tidal cycles), the carbaryl level had declined to 3.3 ppm, over 20 times the EC_{100}. 1-Naphthol levels, however, declined dramatically to 0.5 ppm, a concentration at which symptoms of intoxication probably would not be evident. Carbaryl could be detected down to 6 in. in the mud at 6 wk post application -- 0.1 ppm in the top 1 in., 0.2 ppm at 2-3 in. and 0.08 ppm at 4-6 in. At all of these levels most shrimp would show impairment since these levels range from just below the EC_{50} to just above the EC_{100}. 1-Naphthol could only be detected for 16 days at very low concentrations.

The field study suggests that 1-naphthol would not remain in the environment at high enough concentrations to affect the ability of ghost shrimp to survive. However, carbaryl which remained at or above the 24 hr EC_{100} level for several weeks would have potentially lethal consequences. Carbaryl poisoning, especially at low concentrations, may not lead to the death of ghost shrimp

outright, but changes in behavior and metabolism may leave them unable to cope with a stressful environment.

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APPENDIX

Table 5: Comparison of the oxygen consumption rates ($\mu l\ O_2/g/hr$) of male and female ghost shrimp. Values are means ± 1 SEM. Number of animals is in parentheses. No significant differences were found using a one-way analysis of variance.

Time after exposure (hr)	Sex	Control	Carbaryl	1-naphthol
2.5	M	22.2±1.7 (11)	42.3±3.2 (11)	30.0±5.1 (9)
	F	23.6±3.6 (5)	49.4±7.6 (4)	31.2±4.1 (7)
3.0	M	20.4±1.4 (11)	46.9±3.8 (9)	35.5±4.6 (8)
	F	24.5±2.4 (5)	50.0±7.3 (4)	34.1±4.9 (7)
3.5	M	18.6±1.6 (6)	-----	46.3±5.0 (8)
	F	19.4±1.5 (4)	-----	37.0±5.3 (6)
4.0	M	22.0±2.7 (11)	52.2±4.9 (11)	64.6±6.3 (9)
	F	25.9±4.2 (5)	55.7±8.5 (4)	46.9±6.5 (7)

Table 6: The relationship between weight (log) and oxygen consumption rate (µl O_2/g/hr; log). A regression coefficient which is not significantly different from 0 indicates that the rate is not affected by size of the organism.

Treatment	Correlation Coeff. (r)	Slope (b)	Significance
Control	-0.108	-0.066	ns
Carbaryl	-0.206	-0.191	ns
1-Naphthol	-0.049	-0.053	ns

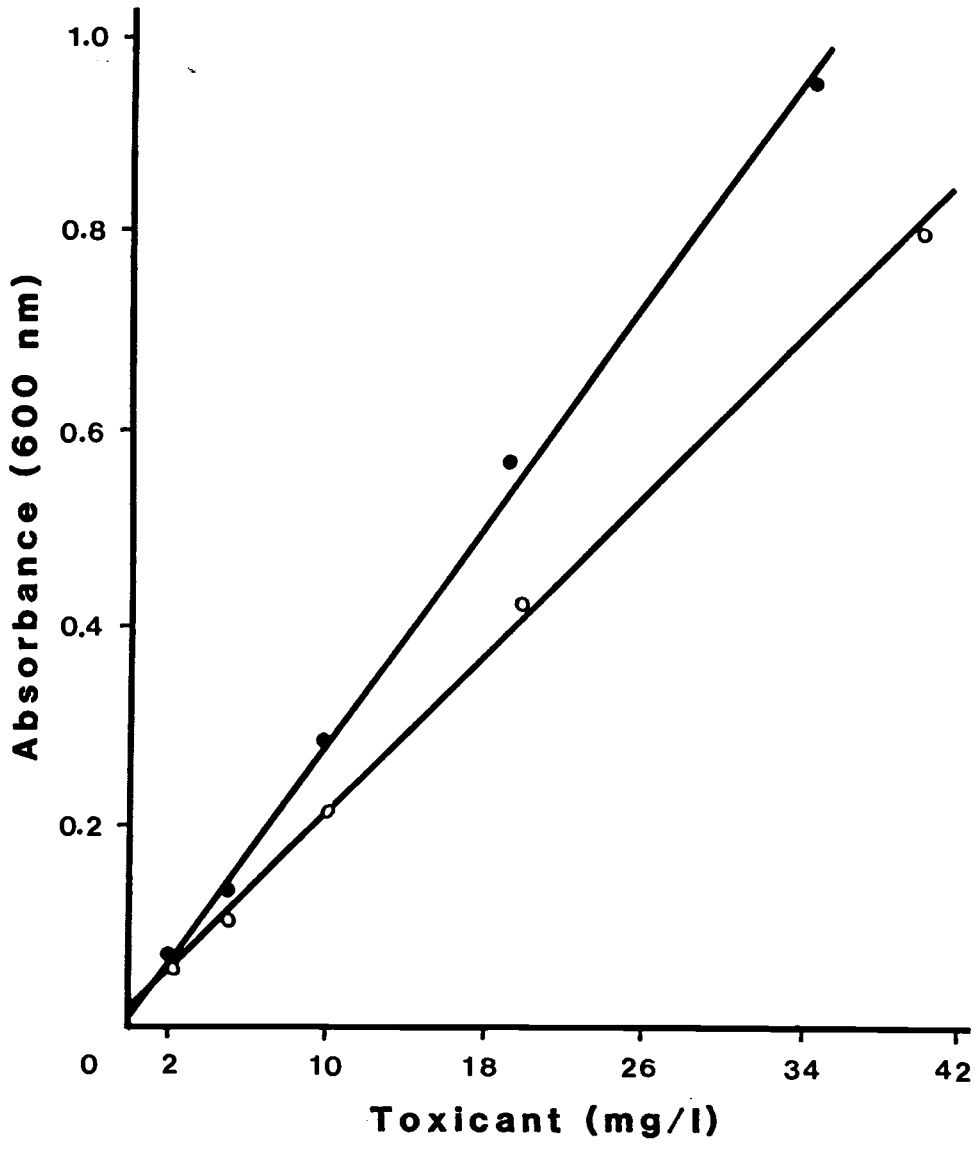

Figure 18. Standard curves for carbaryl (o) and 1-naphthol (●) used in the colorimetric assay.