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Full Length Research Paper

Effects of parathion on cardiac rate, ventilatory frequency and hemolymphatic gas levels in the estuarine crab *Neohelice granulata* (Decapoda, Brachyura)

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Adult crabs *Neohelice granulata* were exposed for 96 h to 0.125 mg/L of parathion under complete submersion. Crabs were then exposed to air for 6 h (emersion period) to be finally re-submerged. Cardiac rate and ventilatory frequency, as well as hemolymphatic pH, pCO_2 and pO_2 were measured at different times throughout the experiment. Cardiac rate was significantly lower in crabs exposed to parathion, but only during the submersion periods, when cardiac activity is normally high. The insecticide could be affecting the neurological regulation of heart, avoiding the normal increase of cardiac activity. Ventilatory frequency, hemolymphatic, pH and gas parameters remained unaltered by exposure to 0.125 mg/L parathion, both under submersion and emersion.

Key words: Parathion, cardiac rate, crabs, submersion, emersion.

INTRODUCTION

The South American crab *Neohelice* (formerly *Chasmagnathus*) *granulata* (Decapoda, Brachyura, Varunidae) is an intertidal, semiterresterial species, widely distributed along the coast of Argentine and Brazil. As stated by Spivak (2010), this species has been extensively studied during the last 25 years, becoming a reference model for both physiological and toxicological research.

Intertidal crabs are daily exposed to the tidal flux, therefore being alternatively exposed to air and water. *N. granulata* actually breathes in both media; thus, several interacting mechanisms are involved for gas exchange and distribution, as in other crab species (Burnett, 1988; Truchot, 1990; McMahon, 2001). Besides, gas exchange, circulation, ionic regulation and acid-base balance are processes strongly inter-related in crustaceans, especially in intertidal crabs (Böttcher and Siebers, 1993; Burnett, 1988; Bianchini et al., 2008). Since heart rate is influenced by several physiological processes, such as ventilation, excretion and osmoregulation, it has a direct relevance for both the organism performance and population health (Depledge et al., 1995). In several previous studies made on crustaceans, heart rate has been used as a physiological biomarker of pollution (Depledge et al., 1995; Lundebye et al., 1997; Brown et al., 2004). An increased heart rate was observed in the crab *Carcinus maenas,* exposed to copper (Bamber and Depledge, 1997; Camus et al., 2004). On the contrary, cardiac frequency decreased in *C. maenas* exposed to the organophosphate insecticide dimethoate (Lundebye et al., 1997).

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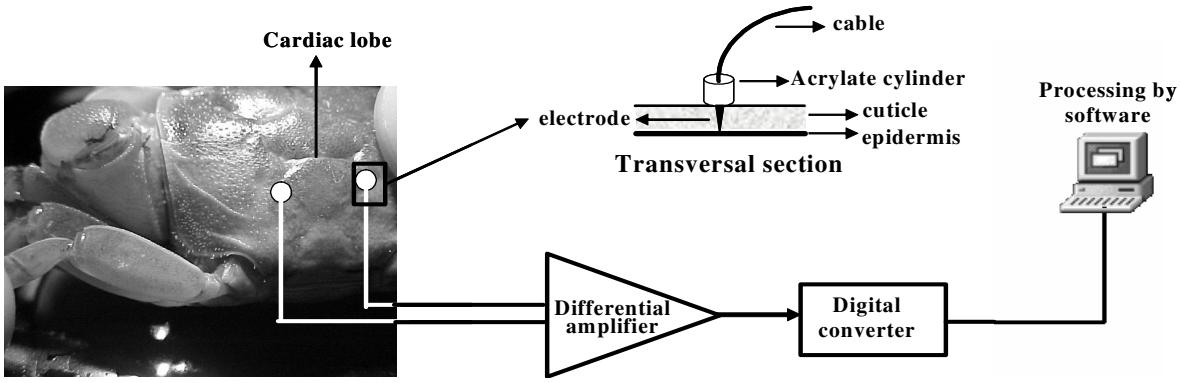

Figure 1. Diagram of the electrodes used for measuring cardiac rate. Data acquisition and processing are also indicated.

Parathion is an organophosphate insecticide extensively used in Argentina since several decades ago. It was detected above the permissible levels in the "Río de La Plata" estuary and its main affluent is the Paraná River (Administrative Commission of Río de La Plata, 1990; Lenardon et al., 1984). The mode of action of this highly toxic pesticide is based on the inhibition of the enzyme acetylcholinesterase (Verhaar et al., 1992). The toxicity of parathion and other organophosphate pesticides have been studied in several species of fish and crustaceans (Van der Oost et al., 2003; Hanazato, 2001; Rodríguez et al., 2007). In the studied species, the lethal toxicity of parathion, as well as several of their sublethal effects, especially on reproduction, has been previously reported (Rodríguez and Lombardo, 1991; Rodríguez et al., 1992; Rodríguez and Pisanó, 1993; Rodríguez et al., 1994; Bianchini and Monserrat, 2007). However, no studies concerning the effects of pesticides on the respiratory gas exchange or circulatory physiology have been made in the studied species, although some studies on the effects of heavy metals on several hemolymphatic parameters have been made (Rodríguez et al., 2001).

The current study was aimed at evaluating the effects of parathion on the ventilatory and cardiac frequencies of adult crabs *N. granulata* alternatively submerged and emerged. Some relevant hemolymphatic parameters, such as hemolymphatic pH, pCO_2 and pO_2, were also measured.

MATERIALS AND METHODS

Adult male crabs (mean wet weight = 13.85 ± 2.07 g, n = 100) were collected at Faro San Antonio beach, located at the southern end of the Samborombón Bay (36°18'S, 56°48'W). After carefully transporting the animals to the laboratory, they were maintained for two weeks at the same environmental conditions to be used later for bioassays: 12L:12D photoperiod (fluorescent light), temperature 20 ± 1°C, pH 7.4 ± 1 and salinity 12 g/L prepared from dechlorinated tap water (total hardness: 80 mg/L as $CaCO_3$ equivalents) and salts for preparing artificial marine water (HW Marine Mix®). During the 2-week acclimation period, crabs were fed twice a week with commercially available pellets of rabbit food (Cervino et al., 1996).

Parathion stock solution at 1 g/L was made from pesticide technical grade (purity 99.9%, Compañia Química, Argentina). Pentaethylene nonylphenolate was used as solvent carrier, in equal proportion to the pesticide, before adding distilled water. This solvent has been used in previous studies, showing no lethal or sublethal effects on the studied species, at concentrations similar or even higher than that used in the current study (Rodríguez and Lombardo, 1991; Rodríguez et al., 1992, 1994; Rodríguez and Pisanó, 1993). A small aliquot of the stock solution was then diluted in 12 g/L saline water to achieve a final pesticide concentration of 0.125 mg/L, this concentration representing 25% of the parathion 96 h-LC50 for *N. granulata* adults, which were determined by Rodríguez and Lombardo (1991) as the incipient lethal threshold concentration for the studied species. A water dilution control was run, containing only the artificial saline water previously specified. A solvent control was also run, containing the solvent concentration presented in the parathion concentration. Ten crabs
were randomly assigned to each treatment (parathion or controls), placing them in a glass aquarium of 24-L capacity filled with 3 L of 12 g/L saline water, prepared as previously specified. All solutions were renewed every 48 h. Crabs were exposed for 96 h to all solutions tested, following the specific procedure previously outlined by Rodríguez and Lombardo (1991).

After the 96-h exposure to parathion, every crab from each treatment was placed in the experimental device. It consisted of several single plastic containers with a plastic mesh screen floor separating the crabs from the bottom. In each container, a single crab was submerged in 500 ml of test solution (water dilution, solvent or parathion 0.125 mg/L), under continuous aeration. The bottom of each recipient was connected to a water reservoir by means of a plastic tube. By changing the relative position of the reservoir, the crab-recipient was slowly filled or emptied out, avoiding stress of animals.

The heart beats were recorded from the bioelectrical activity of the cardiac muscles fibbers, by means of two electrodes implanted in the carapace (Figure 1). This procedure allowed recording the electrocardiogram (EKG) in both submerged and emerged crabs. Each electrode was built with stainless steel wire (diameter 0.5 mm) included in a dental acrylate cylinder (diameter 5 mm, height 5 mm) leaving 1 mm of free-tip, and soldered to an electrically isolated

copper cable. To fix the electrodes, crabs were cold-anesthetized and the pericardial membrane exposed, but not damaged, by carefully drilling through the dorsal carapace. The two active electrodes were adhered with cyanoacrylate glue at each side of cardiac lobe, while a third electrode was fixed in the gastric lobe and taken as ground reference.

The ventilatory frequency was determined by the movements of the scaphognathite (SGM), recorded by means of two copper wires (0.3 mm wide), electrically isolated with exception of the tip. The wires were introduced in the left branchial chamber, through the exhaling channel, until the electrode's tips touched the base of the scaphognathite.

For both cardiac rate and ventilatory frequency recordings, the free ends of the cables were connected to a differential amplifier, AC coupled (Figure 1). The transducer signals were amplified and filtered (bandpass 1 to 40 Hz). Data were acquired and digitized with a sample frequency of 64 Hz, and later processed by Rhythms (Stellate Systems®) software.

All the measurements were performed in a soundproof room, built as a Faraday cage and maintained at 20 ± 1°C. All recordings started at 10 AM. Crabs were kept under complete submersion in the apparatus during 1 h before starting the records, to acclimate them to the experimental device, according to previous studies (Cervino et al., 1995). A first recording of EKG and SGM was made at this initial submersion (SUB1). Next, the test solution was drawn off and the crabs were exposed to air for 6 h, the expected duration of low tide in the natural environment of the studied species. During this emersion period (EM), records were taken at 15, 30, 45, 60, 90, 120, 180, 240, 300 and 360 min. Finally, the recipients of crabs were completely re-filled with the corresponding test solution, and new records were taken at 15 and 30 min after re-submersion (SUB2). In all cases, the duration of each record was 5 min. The cardiac and ventilatory frequencies were determined by counting the wave-spike complex of EKG and waves of SGM in a minute.

A replicate of all treatments was assigned for determination of hemolymphatic pH and partial pressures of gases (pCO_2 and pO_2). Hemolymph samples were taken from 10 different crabs at the base of the fourth pereiopods by means of a capillary tube, at the same times previously specified; pH, pCO_2 and pO_2 were analyzed by means of a Radiometer BMS3 Mk2 Blood micro system held at 20°C.

Two-way ANOVA (treatment, considered as independent factor, and period, considered as a repeated measure factor) was applied to compare mean values. Tukey´s test was used for multiple comparisons of means (Sokal and Rohlf, 1981). A 5% confidence level was always considered.

RESULTS

No mortality was recorded in any treatment, during the 96-h exposure period. Mean values of both cardiac (CF) and ventilatory (VF) frequencies for each considered period (SUB1, EM and SUB2) are shown in Figure 2A and B. Due to their similarity, data from 0 to 60 min of the emersion period were averaged (emersion 1 period: EM1), while the same calculation was applied to data from 90 to 360 min (emersion 2 period: EM2).

Crabs from both water dilution control (WDC) and solvent control (SC) showed, during the EM2 period, a CF significant ($p < 0.05$) lower than during both submersion periods. This pattern was considered as the normal response. On the contrary, CF of crabs exposed to parathion remained constant throughout the experiment. During both the SUB1 and SUB2 periods, a significantly ($p < 0.05$) lower CF was detected in parathion-exposed crabs compared with either WDC or SC crabs, while no differences ($p > 0.05$) were observed between parathion and either control during both emersion periods (Figure 2A).

No significant differences ($p > 0.05$) between either control or parathion-exposed crabs were noted in the VF throughout the experiment (Figure 2B). For all the treatments, a significant ($p < 0.05$) decrease was observed at the beginning of the EM1 period (Figure 2B). The VF values of the SUB2 period were similar ($p > 0.05$) to those of the SUB1 period. No significant differences ($p > 0.05$) were noted between treatments or between exposure periods, in the hemolymphatic pH, pCO_2 or pO_2, at any considered time (Figures 2C to E).

DISCUSSION

The value of cardiac rate for *N. granulata* control crabs during submersion (about 130 beats/min) was similar to that of the aquatic crab *Callinectes sapidus*, measured at 22 to 25°C (DeFur and Mangum, 1979). Besides, a significant bradycardia developed in *N. granulata* control crabs exposed to air, but just after 60 min of exposure. This later result was in accordance with previous reports made for crabs that typically live in aquatic and intertidal environments (Burggren and McMahon, 1988). The bradycardia observed in *N. granulata* was also in accordance with the metabolic depression suggested for the same species during emersion, concomitant with a reduction in both O_2 consumption and locomotive activity (Schmitt and Santos, 1993).

A significant decrease in cardiac frequency was noted in submerged crabs exposed for 96 h to 0.125 mg/L of parathion, compared to controls. Moreover, this decrement was not associated with changes in ventilatory frequency, pH or partial pressures of gases. Therefore, a direct effect of parathion on cardiac physiology seems to be plausible. A correlation between the inhibition of acetylcholinesterase activity and the reduction of heart rate was observed in the crab *C. maenas* exposed to the organophosphorous pesticide dimethoate, suggesting that a disruption in the normal nervous control of heart is taking place (Lundebye et al., 1997).

In crustaceans, the nervous regulation of cardiac rate involves several pathways. First, the neurons of cardiac ganglion, attached to the neurogenic heart, are acting as pacemakers, some of them being cholinergic (Lundebye et al., 1997). Second, both excitatory and inhibitory nerves have been described to innervate the heart (McMahon, 2001), and third, a cholinergic pathway originated from thoracic ganglion regulates the secretory activity of the pericardial organ (Atwood, 1982). Several

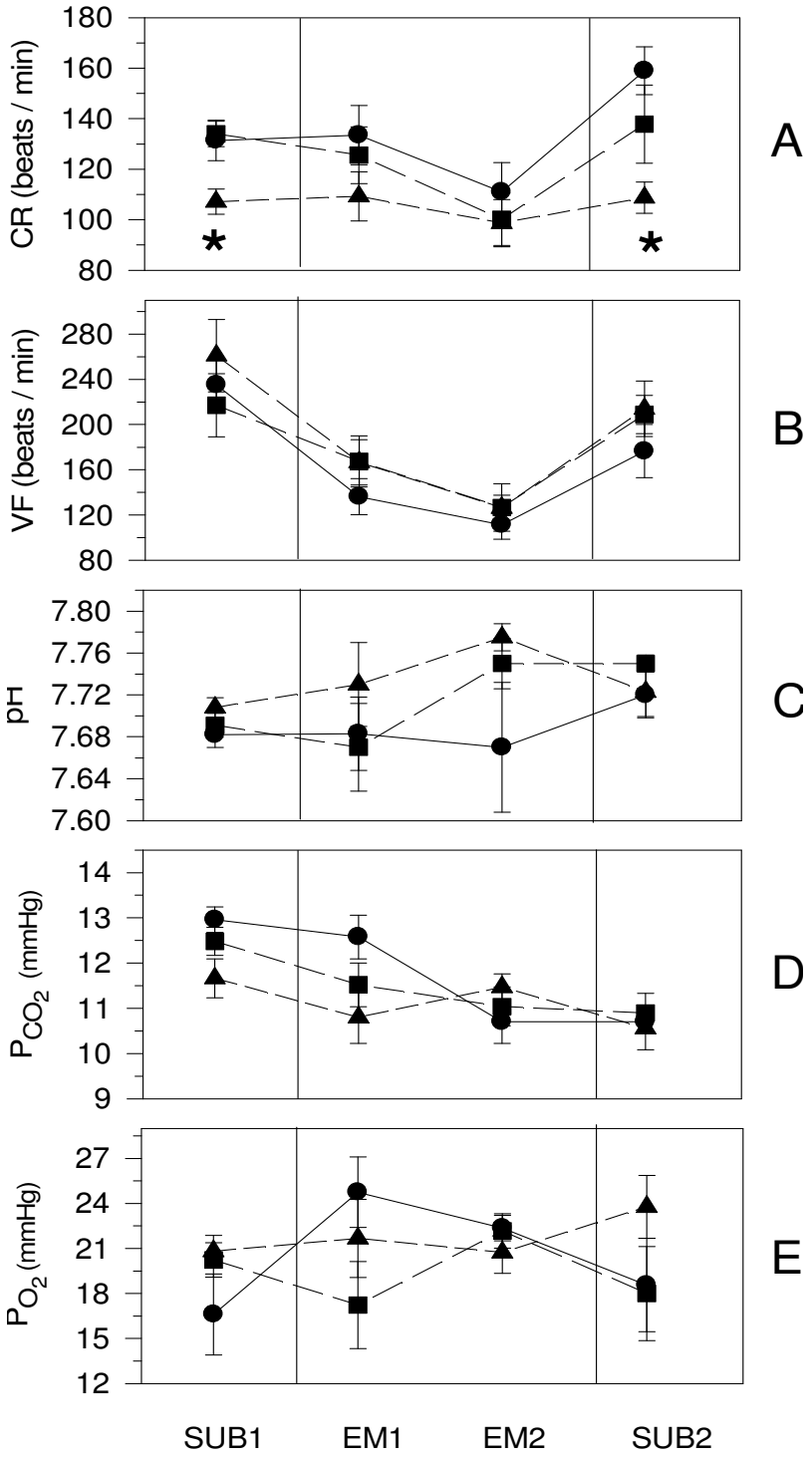

Figure 2. Mean values (± standard error) of (A) Cardiac rate (CR), (B) Ventilatory frequency (VF), (C) pH, (D) PCO_2 and (E) PO_2 during submersion and emersion periods. Each mean represents ten crabs. SUB1: First (initial) period of submersion; EM1: First period of emersion (0 to 60 min); EM2: Second period of emersion (90 to 360 min); SUB2: Second (final) period of submersion. Circles: Water dilution control; squares: Solvent control; triangles: Parathion at 0.125 mg/L. Asterisks indicate significant differences ($p < 0.05$) between parathion and either water dilution or solvent control.

cardioexcitatory amines are secreted by the pericardial organ of crabs, such as serotonin, octopamine and also the crustacean cardioactive peptide, all of them with a stimulatory effect on cardiac rate (Atwood, 1982; Wilkens, 1987; McMahon, 2001). One or several of these pathways could be affected by the anticholinesterase effect of parathion, producing an impairment of the normal heart rate, which becomes evident when it is to rise during submersion. The effect of parathion of reducing CF would therefore be relevant when, for instance, the mentioned biogenic amines are being actively secreted, that is, during the submersion periods, but not during emersion, when CF normally decreases.

Depledge et al. (1995) have suggested that physiological variables measured on crabs, such as heart rate and ventilatory frequency, can be potentially used as biomarkers of pollution, since they are integrated responses which reflect the overall performance of the animal, also showing to be sensitive under the exposure to several pollutants. As instance, Bamber and Depledge (1997) have reported a consistent increase in the heart rate of the crab *C. maenas* exposed to copper, suggesting that such increased rate is related to respiratory stress, rather than to a direct effect of copper on heart. The increase of heart rate caused by copper has been also reported, in the same species, to be enhanced by either low or high temperature (Camus et al., 2004). An increased heart rate during daytime was also reported for both the crab *Potamon potamios* and the crayfish *Astacus astacus* exposed to mercury (Styrishave and Depledge, 1996).

Concerning hydrocarbons, no significant effects on heart rate of adult *C. maenas* have been detected at either relatively high doses of benzo[a]pyrene (Bamber and Depledge, 1997), or pyrene (Dissanayake et al., 2008). As for pesticides, Lundebye et al. (1997) have reported, as mentioned before, a decrease in heart rate of crabs *C. maenas* exposed to the organophosphorous insecticide dimiethoate, in correlation with the inhibition of acetylcholinesterase. Our results with parathion also associate a decreasing heart rate in submerged crabs with an organophosphate intoxication, supporting the idea that this physiological variable could be a specific biomarker for identifying the exposure to acetylcholinesterase inhibitors. In this respect, since parathion exerts an irreversible inhibition on acethylcholinesterase, further assays to determine the degree and time of reversion for heart rate of crabs transferred to clean water would be relevant.

Gill damage was observed in adult crabs *N. granulata* exposed during 96 h to 0.25 mg/L of parathion; concomitantly, hemolymphatic pCO_2 and lactate were increased due to the impairment of CO_2 and O_2 exchange at gills, leading to a blood acidosis of both respiratory and metabolic origin (Medesani et al., unpublished). In the current study, though, the hemolymphatic pH and partial pressures of gases remained unaffected from one treatment to another, regardless of the submersion state and the exposure to 0.125 mg/L of parathion. The insecticide did not cause changes in ventilatory frequency in any case, in good agreement with absent of effect observed on the hemolymphatic parameters. Therefore, the current study has evaluated the effect of parathion on heart rate and ventilation, at a concentration that allowed discarding the possible modifying effect that hemolymphatic gas level and/or pH could have on those physiological variables.

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