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Short communication

Differential immune responses of the green neon shrimp (*Neocaridina denticulate*) to dipropyl phthalate

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ABSTRACT

This study set out to understand the sublethal effect of xenobiotic phthalate esters (PAEs) on the relationship between the susceptibility of shrimp to bacterial infection and the immune response of the shrimp. *Neocaridina denticulate* were exposed to different concentrations of the PAE dipropyl phthalate (DPrP), and mortality and six immune parameters were measured on days 1, 3, 5, and 10 after exposure. On days 1 and 3 after exposure, shrimp exposed to 0, 1, 5, 10, and 50 mg/L of DPrP and challenged with *Aeromonas veronii* experienced 14% and 16%, 16% and 16%, 18% and 18%, 34% and 24%, and 38% and 26% mortality, respectively. On day 1, five immune parameters (acid phosphatase, AcP; β-glucuronidase, β-Glu; phenoloxidase, PO; superoxide dismutase, SOD; and haemocyanin mRNA) were significantly altered in the all of the groups treated with DPrP compared to the untreated shrimp and were elevated in the 10 mg/L- and 50 mg/L-treated groups. Beta−Glu activity and haemocyanin mRNA levels were significantly increased in a dose-dependent manner. These results suggest that the increased susceptibility of *N. denticulate* exposed to DPrP is short-term and may be related to the increased expression of DPrP-induced immune mediators.

1. Introduction

Increasing evidence indicates that many xenobiotics, a product of chemical pollutants that are degraded but not biologically decomposed in sewage treatment systems, are often not acutely toxic for exposed aquatic animals but instead lead to chronic intoxication resulting in tissue alterations. There is a developing awareness that diseases in both fish and mollusk populations are linked to environmental changes or coastal marine pollution. Considerable evidence exists supporting links between environmental changes, including contaminants, non-infectious diseases and a depression of the immune system [1,2]. In crustaceans, environmental stress from pollutants such as polychlorinated biphenyls, polynuclear aromatic hydrocarbons, and heavy metals seems to be an important factor in determining the level of immune suppression, indicated by the appearance or increased prevalence of disease in populations exposed to these pollutants [3,4,5,6].

Phthalate esters (PAEs) are widely used industrial chemicals that serve as important additives to impart flexibility to polyvinyl chloride (PVC) resins and have become widely diffused in the environment [7] via the manufacturing process. In Taiwan, PAEs have been found to be widely distributed in river water, sediment, and soil [8] and have additionally been found to accumulate in fish [9]. Numerous experiments have shown that the bioaccumulation of PAEs occurs in aquatic and terrestrial food chains due to their low solubility and degradation combined with their high hydrophobicity and adhesion [7]. This accumulation interferes considerably with the propagation and size of animal populations as a result of abnormal sexual development and a reduction in reproductive functions [10]. In addition, PAEs are likely to be a part of a mechanism responsible for embryonic toxicity in abalone [11]. The United States Environmental Protection Agency (USEPA) and its counterparts in several other countries have classified the most commonly occurring PAEs as priority pollutants and endocrine disrupting compounds. Previous studies conducted by our group demonstrated that PAEs, including dipropyl phthalate (DPrP), could damage haemocytes of an economically important freshwater prawn species (*Macrobranchium rosenbergii*) and could result in an increase in susceptibility of these prawns to pathogen infection [12,13]. Therefore, it is of particular importance to evaluate the effects of PAE pollution in the environment and its toxicity to aquaculture organisms.

Neocaridina denticulate (De Haan. 1844, Crustacea, Decapoda) is found in rivers throughout eastern Asia and the Hawaiian islands and is also a common shrimp in the freshwater ecosystems of Taiwan. Several characteristics of *Neocaridina denticulata* are

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beneficial as aquatic indicators to assess environmental pollution. The shrimp has been used in few studies to determine the effects of pollutants, such as chlordane and lindane, on growth and reproductive hormones [14]. In 2005, the Environmental Protection Administration of Taiwan announced this organism is one of indicators used in the acute toxicity assay to assess whether various water bodies or effluents from industrial districts are qualified. The purpose of this study was to examine the effects of exposure to different concentrations of DPrP for 10 days on *N. denticulate* mortality and six parameters related the animal's immune functions, including three intrahaemocytic lysosomal enzymes involved in phagocytosis (acid phosphatase, AcP; α-naphthyl acetate esterase, ANAE; and β-glucuronidase, β-Glu), one antioxidant enzyme (superoxide dismutase, SOD), one defence-related enzyme (phenoloxidase, PO), and the immunity- and metabolism-related protein haemocyanin. The results from this study indicate the effects of sublethal dosages of DPrP on the immunity of *N. denticulate* and may be useful in assessing the feasibility of employing *N. denticulate* as an aquatic indicator for biomonitoring aquacultural water systems.

2. Materials and methods

2.1. Shrimp acclimation

Shrimps (*N. denticulate*) with body lengths of 2–3 cm/individual were acclimated in 20-L glass aquaria containing fresh pond water and water plants (*Egeria densa* Planch) at 28 °C, pH 6.9–7.5, oxygen concentration > 4 mg/L and a 12 h light–dark photoperiod. The shrimp were acclimated for at least one week prior to the experiments. The shrimp were fed with mosquito larvae twice a day, in the morning and at the nightfall. The stocking densities were maintained at 100 individuals per aquarium.

2.2. Immersion treatment with DPrP

Dipropyl phthalate (DPrP, Chem Service Co.) was dissolved in acetone to a concentration of 50,000 mg/L as a stock solution and stored at room temperature. Prior to immersion, the stock solution was serially diluted into different concentrations with fresh pond water. Preliminary tests had shown that the mortalities of DPrP-treated shrimp subjected to concentrations of 10, 50, and 100 mg/L continuously for 10 days were 2%, 6%, and 15%, respectively; mortality of the control (acetone-treated group) was less than 2%. Therefore, in these experiments, shrimp were continuously treated with DPrP at four concentrations (1, 5, 10, and 50 mg/L) with a density of 70 individuals/L for 10 days. The control shrimp were treated with pond water containing a 1000-fold dilution of acetone. During the experimental period, a third of pond water was displaced every day with the fresh-prepared pond water containing different concentration of DPrP.

2.3. Challenge experiment

The bacterial strain *Aeromonas veronii*, which was originally isolated from the hepatopancreas of giant freshwater prawns [15], was used for experimental challenges. The bacterial suspension was prepared according to procedures described by Sung et al. [16]. In the challenge experiment, shrimps were divided into seven groups with 250 individuals in each group. Four experimental groups were bathed continuously with different concentrations of DPrP (1, 5, 10, and 50 mg/L of pond water). Three control groups were used. One control group (control group 2) was immersed in pond water containing 50 mg/L DPrP, and the other two groups (control group 1 and 3) were immersed in pond water containing 0.1% (v/v) acetone. On days 1, 3, 5, and 10 after treatment, 50 shrimp from either each of the four groups or control group 1 were immersed in 10^9 bacterial cfu/L of pond water, and the control group 2 and 3 (DPrP- and acetone-treated) were treated with pond water. After immersion, shrimps were held in aquaria at 28 °C with aeration. The number of dead shrimp was recorded twice daily until 2 days had elapsed with no shrimp dying. Mortality percentages were calculated using the following formula: (total number of dead shrimps - number of non-specific death)/(total number of shrimps - number of non-specific death) × 100%, where non-specific death represents the number of deaths caused by the stress of the operating process within the first 12 h after immersion.

2.4. Preparation of haemocytes and tissue lysate supernatants

For assays of enzyme cytochemistry, formalin-fixed haemocyte samples were prepared according to procedures described by Sung and Sun [16]. Fixed haemocytes were collected from haemolymph with an artificially capillaceous needle rinsed with anticoagulant (0.01 M Tris–HCl, 0.25 M sucrose, 0.1 M tri-sodium citrate, pH 7.6). In addition, because the size of the individual shrimp was too small to collect sufficient haemocytes for different assays, tissue lysate supernatants (TLS) were prepared from five individuals and used separately in examining the activities of two enzymes (PO and SOD). Briefly, after the shrimp were immersed in ice-cold PBS (0.01 M, pH 7.0) for 1–2 min, they were ground into powder using a liquid nitrogen-chilled mortar and a Teflon pestle (Kontes, USA). Afterward, the tissue homogenate was suspended in ice-cold 25 mM tris–HCl buffer with 25 mM EDTA and then centrifuged at 12,000 × *g* for 15 min at 4 °C. The protein concentration of the resultant TLS was determined using a Bio-Rad Protein assay Kit II (Hercules, USA).

2.5. Enzyme cytochemistry

Prior to assays, fixed-haemocyte samples prepared from individual shrimp were spread onto three separate glass cover slips that were treated with 0.2% poly-L-lysine solution and air-dried. The expression of three intrahaemocytic lysosomal enzymes (AcP, ANAE, β-Glu) was assayed using commercial kits 91-A, 81-A, and 181-C according to the procedures recommended by the manufacturer (Sigma, USA) and microscopically observed after incubation at 37 °C for 1 h. The number of positive haemocytes with enzyme activity in each individual was counted by observation of a minimum of 50 randomly selected haemocytes under a light microscope. The percentages of positive cells were calculated using the formula: (the number of positive haemocytes/the total number of haemocytes) × 100. The enzyme detection data were expressed as a ratio with respect to the controls, which was calculated as the percentage of DPrP-treated shrimp/the percentage of untreated shrimp.

2.6. Assay of PO and SOD activities

The PO activity was examined as described by Sung et al. [16]. One unit of enzyme activity was defined as an increase in the absorbance of 0.001 min/mg of protein. The value resulting from the experiment was the relative PO activity (RA_{PO}), which was calculated using the formula: PO of TLS from DPrP-treated group/PO of TLS from untreated group. For the assay of SOD activity, the activities of both TLS and standard SOD solutions containing different known concentrations were used to determine the inhibition percentage to construct a reference standard curve of SOD activity, as determined using a Ransod Kit (Randox, UK), based on the inhibition of superoxide radical-dependent reactions, according