ORIGINAL RESEARCH

Effects of Chlordane and Lindane on Testosterone and Vitellogenin Levels in Green Neon Shrimp (*Neocaridina denticulata*)

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The purpose of this study was to investigate the acute toxicity of chlordane and lindane as well as their endocrine disruption effect on green neon shrimp (*Neocaridina denticulata*), a common habitant in freshwater system of eastern Asia and Hawaii. First, the organisms were exposed to chlordane and lindane to estimate the 96-h LC_{50}(96-h median lethal concentration). Then, levels of testosterone and vitellogenin in hemolymph of *N. denticulata* after exposure to sublethal concentrations of chlordane (1 ng/L and 10 ng/L) and lindane (0.1 μg/L and 1 μg/L) were also examined. The 96-h LC_{50} values obtained from the results of acute exposure were 127.03 (130.11–122.35) ng/L and 9.36 (8.00–10.96) μg/L for chlordane and lindane, respectively. Furthermore, reductions of testosterone concentration were observed in both chlordane- and lindane-treated shrimps, whereas induction of vitellogenin-like protein was only apparent in chlordane-treated shrimps. Thus, it is concluded that chlordane and lindane may probably show some disruption endocrine functions on *N. denticulata*.

Chlordane and lindane, being organochlorine pesticides (OCPs), have been extensively used over the last two decades. OCPs are some of the most dangerous pesticides due to their toxicity, stability, high liposolubility, and long biological half-life. OCPs exhibit a high degree of bioaccumulation and biomagnification within the food chain, and these chemicals are known to be carcinogenic, teratogenic, and have endocrine disrupting activity in humans and wildlife (Klaassen 2001). Therefore, many developing and developed countries have prohibited the use of

these compounds for many years, even though OCPs are still being detected in ecosystems (Chen, Wang, and Yaun 1999). Both chlordane and lindane are reported to have symptoms similar to endocrine disrupting chemicals (EDCs) (Colborn, vom Saal, and Soto 1993). In animal studies, these compounds have been shown to actually mimic estrogen (xenoestrogen) and to cause detrimental effects on reproductive systems (Colborn, vom Saal, and Soto 1993; Keith 1997). The US Environmental Protection Agency (EPA) has designated that chlordane is a probable human carcinogen, causing hepatic tumors as well as acute leukemia in mammals. Prenatal mice exposed to chlordane exhibited immunotoxic effects and caused uterus toxicity (Keith 1997). Much literature exists showing that lindane exerts neurotoxic, hepatotoxic, and uterotoxic effects (Keith 1997).

Invertebrates (especially arthropods such as insects and crustaceans) constitute the vast majority of animal species on earth, and represent an important and diverse group within the crustaceans. The populations of crustaceans often rely on the water quality and food supply. Consequently, their populations also influence the fish production in ecosystem (Dodson 1989). Therefore, examining the ecological characteristics of crustaceans will facilitate our understanding of ecology, because of their key positions in communities (Dodson 1989; Dodson, Hanazato, and Gorski 1995). Due to the increasing serious water pollution, the environment also contains anthropogenic toxicants, which affect the biota in complex and synergistic ways. Although toxicity test protocols of many invertebrate are routinely used in regulatory toxicity testing, few have been designed with endocrine-specific end point in mind. The objective of this study was to examine the toxicity of chlordane and lindane in green neon shrimp (*Neocaridina denticulata*), a species extensively distributed in rivers throughout eastern Asia and Hawaiian Island (Englund and Cai 1999; Hung, Chan, and Yu 1993). Our hypothesis was that testosterone and vitellogenin (Vg) levels in the bodies of *N. denticulata* can change after exposure to chlordane and lindane to show their endocrine disrupting function.

MATERIALS AND METHODS

Animal Collection and Maintenance

Green neon shrimp (*N. denticulata*) of 9 to 12 mm body length were taken from rivers in Taipei County for laboratory testing. They were transferred to a 50-L glass aquarium after being identified. This aquarium was equipped with a water-cycling device, pH was maintained at 7.4 to 7.8, dissolved oxygen concentration was greater than 7.3 mg/L, and hardness was 38 to 45 mg $CaCO_3$/L. Temperature was maintained at 25°C ± 1°C, and a 12-h light-dark photoperiod was used. Under these conditions, shrimp were fed twice a day and acclimated for 2 weeks before testing.

Chemicals and Water Renewal

Chlordane and lindane were purchased from Sigma (St. Louis, MO, USA). A stock solution of chlordane (10 mg/L) and lindane (100 mg/L) was prepared in acetone and stored in refrigeration (4°C) for use. Toxicity tests with water renewal based on the *Standard Guide for Conducting Acute Tests with Shrimps* every 48 hours (EPA/ROC 1998).

Establishment of Median Lethal Concentration

Shrimp were divided into 10 groups (20 shrimp/group) in 5-L glass beakers and exposed to various pesticide concentrations as shown in Table 1. Each treatment was done in triplicate. During the experiment, dead individuals were removed and mortality of shrimp was recorded at the 2nd, 6th, 24th, 48th, 96th, and 120th h. Data obtained in this test were analyzed to estimate the 96-h LC_{50} using the method of Finney (1972).

Assays for Vitellogenin and Testosterone

The 96-h median lethal concentration (96-h LC_{50}) was the pilot study used for determining the pesticide concentrations for chronic studies. Sublethal concentrations of chlordane and lindane were 1 and 10 ng/L and 0.1 and 1 µg/L, respectively. Experiments with positive controls (10 µg/L and 1 mg/L 17β-estradiol in alcohol) and no pesticide added were also carried out, as well as those using a vehicle-control group in which only acetone was added. In total, there were 100 juvenile shrimp (body length 9 to 12 mm) of same size in each group (in a 10-L glass beaker, run in triplicate), and samples were taken at the end of days 1, 3, 7, 14, and 28.

In preparation for vitellogenin-like protein and testosterone analysis, shrimp samples were homogenized with a Teflon pestle (Kontes, Vineland, NJ, USA) in ice-cold 25 mM Tris-HCl with EDTA. The homogenate was centrifuged at 10,000 × g (for 20 min at 4°C), and the supernatant was collected and stored at −20°C until analysis.

The level of vitellogenin-like protein in the hemolymph was mediate determined by using an alkali-labile phosphate (ALP) measurement assay and the quantity of ALP in hemolymph was obtained similar to the method of Gangé and Blaise (2000). Shrimp hemolymph (in 5 and 45 µl of Tris-HCl buffer, 25 mM, pH 7.5) was mixed with 50 µl ice-cold 20% trichloroacetic acid (TCA) and incubated at room temperature for 15 min. The mixture was centrifuged at 10,000 × g for 10 min at 4°C. The protein pellet was resuspended with 200 µl 1 M NaOH and then heated to 75°C for 60 min before determination. The level of free phosphates was determined according to the phosphomolybdenum method, and the optical absorbance was read at 600 nm.

TABLE 1

Pesticide concentrations and green neon shrimp (*Neocaridina denticulata*) mortality (%) in acute toxicity and the 96-h LC_{50}

Pesticide	Average shrimp weight (mg)	Average shrimp length (mm)	Nominal concentration* (µg/L)	Mean mortality (%)	96-h LC_{50} (µg/L)
Chlordane	11.8	10.2 ± 1.4	0	0	0.127
			0.005	10	
			0.01	15	
			0.03	17	
			0.1	22	
			0.3	100	
Lindane	11.9	10.0 ± 3.4	0	0	9.36
			1	0	
			3	5	
			5	10	
			10	55	
			20	90	
			30	100	

*The final concentrations were obtained from acetone solutions.

Levels of testosterone were determined using an EIA Kit (Cayman, Ann Arbor, MI, USA). This assay is based on the competition between testosterone and a testosterone-acetylcholinesterase (AChE) conjugate (testosterone tracer) for a limited number of testosterone-specific rabbit antiserum binding sites (Pradelles, Grassi, and Maclouf 1985; Maxey, Maddipati, and Birkmeter 1992). The product of this enzymatic reaction has a distinct yellow color and peak absorbance at 405 nm detectable by spectrophotometer.

Statistical Analysis

Statistical analysis used Microcal Origin 6.0 software. (Northampton, MA, USA, 1999). Experimental and control values were compared using Student's t test (paired assay, $p < .05$). Areas under the curves indicating quantities during the levels of testosterone testing were obtained via an integration method in Microcal Origin 6.0.

RESULTS AND DISCUSSION

The 96-h LC_{50} values obtained were 127.03 (130.11–122.35) ng/L and 9.36 (8.00–10.96) μg/L for chlordane and lindane, respectively (Table 1). Obviously the toxicity of chlordane to *N. denticulata* is more obvious than that of lindane. So far, there is only limited knowledge of the adverse effects of pesticides on *N. denticulata*. Galindo, Jasso, and Lizarraga (1996) reported that the values of 48-h LC_{50} for chlordane and lindane of 63.2 and 3.9 μg/L for white shrimp (*Penaeus vannamei*), respectively. In fish, Bansal et al. (1980) also demonstrated that 12 to 20 μg/L chlordane was the values of LC_{50} for the larvae of four species of freshwater carp. Therefore, with our results a wide range of toxicity exists for these pesticides in a variety of aquatic organisms as well as within species. However, it is clear that both pesticides exert more toxicity more toxic to shrimp than fish.

Values of hemolymph testosterone concentrations in 17β-estradiol, chlordane, and lindane-treated groups were significantly ($p < 0.05$) lower than those of the control group (Figure 1). This is especially noteworthy in the cases of 10 μg/L and 1 mg/L 17β-estradiol. That is, hemolymph testosterone concentrations showed a rapid drop in 3 days, then rose steadily during 7 to 28 days with treatments of 10 μg/L 17β-estradiol; treatment with 1 mg/L 17β-estradiol produced a slight decrease during the entire experimental period. On the 28th day, testosterone concentrations were both close to 1.8 ng/g with treatments of 10 μg/L and 1 mg/L 17β-estradiol (Figure 1b). Our results show that the areas for the control and treatments with 10 μg/L 17β-estradiol, 1 mg/L 17β-estradiol, 1 ng/L chlordane, 10 ng/L chlordane, 0.1 μg/L lindane, and 1 μg/L lindane were 72.6, 55.5, 48.2, 55.1, 49.7, 52.6, and 49.51 ng/g/28 days, respectively. Thus, areas relative to the control for treatments with 10 μg/L 17β-estradiol, 1 mg/L 17β-estradiol, 1 ng/L chlordane, 10 ng/L chlordane, 0.1 μg/L lindane, and 1 μg/L lindane decreased by 23.6%, 33.6%, 24.1%, 31.6%, 27.6%, and 31.8%, respectively. These data showed that treatment with chlordane
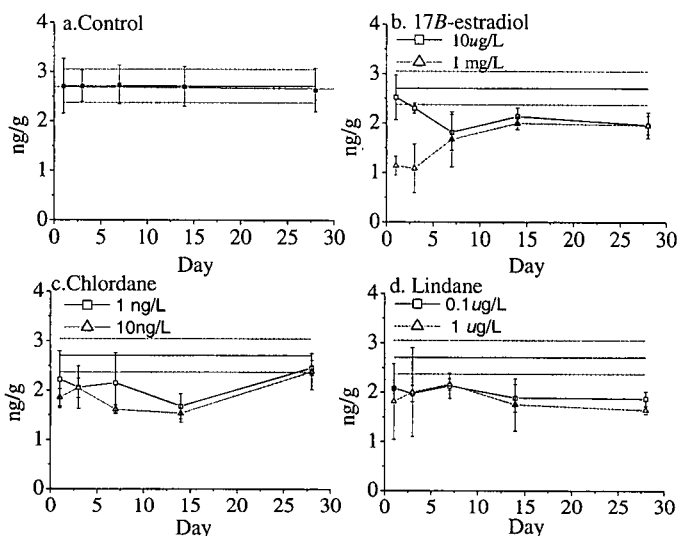

FIGURE 1

Hemolymph testosterone response levels in green neon shrimp exposed to 17β-estradiol (*b*), chlordane (*c*), and lindane (*d*). The solid line shows the mean of control (2.7 ng/g), and dotted line shows 95% confidence limits (3.1, 2.4 ng/g).

and lindane changed the levels of testosterone in the hemolymph, similar to that treated with 17β-estradiol as the positive control. This is good evidence proving that chlordane and lindane can result into the decrease the levels of testosterone in *N. denticulata*. The mechanistic of the decrease in testosterone is unknown. It is possible that the presence of xenoestrogen exert a feedback inhibition effect on the steroidogenesis. Another speculation is that aromatase may be activated to convert testosterone to estrogen.

Treatment with 17β-estradiol, chlordane, and lindane changed the levels of vitellogenin (Vg) in the hemolymph, as determined by the ALP assays. Vg levels in *N. denticulata* hemolymph were induced from treatments with 17β-estradiol and chlordane on day 7; however, there were no significant differences ($p < .05$) with the results from treatment with lindane (Figure 2). However, Vg determined by ALP assays may not reflect accurate measurement of Vg, due to the present of phosphovitin-like protein and phosphovitin in addition to Vg in the hemolymph. However, it could be conceded as simple and time saving way to determine Vg. An enzyme-linked immunosorbent assay (ELISA) assay can be used for the future study.

Our study shows that the testosterone levels of *N. denticulata* can be affected by chlordane and lindane. The male gonad was found to be a highly sensitive target organ for lindane (Silvestroni and Palleschi 1999). This phenomenon was also observed in results of treatments of the lindane group in this study. Although the concentration of testosterone in the chlordane group was initially shown to be inhibited, its level had risen by the 28th day during the experimental period. Therefore, it is concluded that chlordane did not directly affect the testosterone levels of
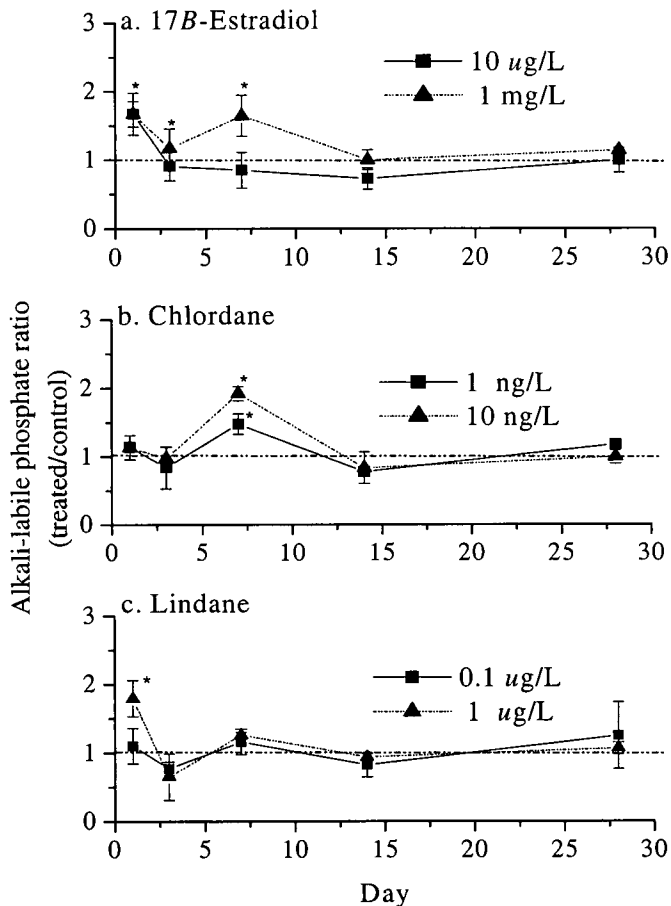
FIGURE 2

Hemolymph APL response levels in green neon shrimp
exposure to 17β-estradiol (*a*), chlordane (*b*), and lindane (*c*).
Asterisk (*) indicates significant differences between
experimental and control at $p < .05$.

N. denticulata. On the other hand, the results of this study
showed that chlordane caused an increase in Vg concentration,
as did 17β-estradiol (the positive control). Under normal cir-
cumstances, a mature female organism usually shows a high
level of estrogen, which can induce the Vg synthesis, whereas
a male or immature individual shows a much lower level of
estrogen, which cannot trigger the synthesis. However, things
become different when the male or immature organism is ex-
posed to the xenoestrogne, that is, Vg synthesis can probably be
induced via the binding of xenoestrogne to estrogen receptors
(Kime, Nash, and Scott 1999). It is speculated that the increased
Vg synthesis may be related to the activation of hepatopancreas
estrogen receptor (Tseng et al. 2001). In our study, we observed
the Vg synthesis in a chlordane-treated group on *N. denticulata*,
and therefore chlordane probably had an estrogenic effect on
N. denticulata. As for lindane, although there was no significant
difference shown in the statistics, this was probably either be-
cause the dose applied could not induce Vg synthesis or because
the lindane had no effect on the estrogen receptor of *N. dentic-*

ulata. Anyhow, our present studies showed that chlordane and
lindane may probably have some disruption endocrine functions
on *N. denticulata.*

Hormones control male and female reproductive systems
(Hasegawa and Hirose 1993; Taylor and Harrison 1999). Lit-
erature on the basic endocrinology of crustaceans, such as crab,
lobster, and shrimp, suggests that estrogens, e.g., 17β-estradiol,
are stimulating hormones for female sexual maturation and egg
laying (Andersen, Halling-Sørensen, and Kusk 1999). How-
ever, low concentrations of chlordane (<14.2 μg/L) and lindane
(<20.6 ng/L) can still be detected in freshwater (Abou-Arab et
al. 1995; Chen, Wang, and Yaun 1999; Galindo et al. 1999).
When chemicals such as chlordane and lindane reach wildlife
through the food chain, they may cause reproductive problems.
As a result, decreases in wildlife populations could be well ex-
pected (Colborn, vom Saal, and Soto 1993). This is an urgency
needing our attention to find out the biologically safe concentra-
tion if the reproductive systems and behaviors of *N. denticulata*
in our environment are not to be changed. We are currently
working on related studies to further understand the effects of
chlordane and lindane on *N. denticulata.*

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