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Effects of the endocrine disrupter chemicals chlordane and lindane on the male green neon shrimp (*Neocaridina denticulata*)

Da-Ji Huang [a], Shu-Yin Wang [b], Hon-Cheng Chen [a,c,*]

[a] *Institute of Zoology, National Taiwan University, No. 1, Sec. 4, Roosevelt Road, Taipei 106, Taiwan, Province of China*
[b] *Department of Animal Science, Chinese Culture University, Yang Ming Shan, Taiwan 111, Province of China*
[c] *Institute of Fisheries Sciences, National Taiwan University, Taipei 106, Taiwan, Province of China*

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Abstract

The purpose of this study was to investigate the effect of chlordane and lindane on morphological changes and endocrine disruption in male Green neon shrimp (*Neocaridina denticulata*). In this experiment, individuals of *N. denticulata*, a common inhabitant of freshwater systems in Taiwan, were exposed to chlordane (1 and 10 ng/l) and lindane (0.1 and 1 μg/l). Morphological changes and reproductive hormone levels were observed following four weeks exposure. According to our findings, an increase in estrogen, a reduction in testosterone, and morphological alternations of the masculine appendage were observed in both chlordane- and lindane-treated shrimp, while induction of a vitellogenin-like protein appeared only in shrimp treated with 10 ng/l chlordane. An endocrine disruption effect on *N. denticulata* was demonstrated, and may apply to other organochlorine pesticides or endocrine disruption chemicals.

Keywords: Endocrine disruption chemicals; Chlordane; Lindane; *Neocaridina denticulata*

1. Introduction

Chlordane and lindane are both organochlorine pesticides (OCPs), are widely distributed contaminants in environments such as aquatic systems, and act as endocrine disruption chemicals (EDCs) to many organisms. OCPs are some of the most dangerous pesticides because of their toxicity, stability, high liposolubility, and long biological half-life. OCPs can exhibit a high degree of bioaccumulation and biomagnification within food chains, and are known to have carcinogenic, teratogenic, and endocrine-disruptive effects in humans and wildlife. Previous studies showed that chlordane may perform complex and variable biological functions such as estrogen-like functions, while lindane has estrogenic-like effects on some organisms, but anti-estrogenic effects on others. Chlordane and lindane have been shown to have similar symptoms to EDCs. In animal studies, these compounds were shown to exhibit an estrogenic-like effect, and to cause detrimental effects to reproductive

* Corresponding author. Address: Institute of Zoology, National Taiwan University, No. 1, Sec. 4, Roosevelt Road, Taipei 106, Taiwan, Province of China. Tel.: +886 2 2363 0231x3324; fax: +886 2 2363 6837.

E-mail addresses: daji@ms19.hinet.net (D.-J. Huang), sywang@faculty.pccu.edu.tw (S.-Y. Wang), honcheng@ccms.ntu.edu.tw (H.-C. Chen).

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systems (Colborn et al., 1993). Although many developing and developed countries have prohibited the use of these compounds for many years, OCPs are consistently detected in ecosystems (Chen et al., 1999). Therefore, the influence of OCPs residues on the development of wildlife has become a major concern.

Several reports have indicated that changes in the reproductive system and morphology of wildlife take place after exposure to xenoestrogen (synthetic, industry-derived estrogenic compounds) (Colborn et al., 1993; Sharara et al., 1998). Crustaceans are frequently used as a target animal in aquatic systems to examine endocrine disruptor chemicals (Depledge and Billinghurst, 1999; Hutchinson, 2002). The physiological role of the "vertebrate type" steroid hormones, testosterone, and estrogen, has been identified in the shrimp (Quinitio et al., 1991; Cardoso et al., 1997). Treatment with natural vertebrate estrogen was reported to induce maturation in crustaceans (Rinderhagen et al., 2000). Several reports showed that the natural hormone and xenoestrogen could effect sexual development and sexual hormone regulation (Colborn et al., 1993; Sharara et al., 1998). Huang and Chen (2004) demonstrated that chlordane and lindane cause an alternation in testosterone and vitellogenin (Vg) levels in juvenile shrimp (*N. denticulata*).

Atyid shrimp are common inhabitants of freshwater systems. In Taiwan, 13 species of atyid shrimp belonging to three genera have been described, among which, *Neocaridina denticulata* is the only one belonging to the genus *Neocaridina* (Hung et al., 1993). This species is commonly and extensively distributed in many streams, ponds, swamps, and rivers in East Asia and the Hawaiian islands (Hung et al., 1993; Englund and Cai, 1999). Thus, *N. denticulata* is one of the most commonly used aquatic organisms for environmentally related studies of freshwater systems (Chen et al., 1999). However, in Taiwan, the quantity of *N. denticulata* in natural habitats is on the decline due to human activities, including pollution, destruction of natural habitats, and overfishing (Shy and Yu, 1998).

Despite having a common distribution, only a few attempts have been made to determine the impacts of chlordane and lindane in freshwater on *N. denticulata*. The purpose of this study was to use the length of the masculine appendage, and levels of vitellogenin, estradiol, and testosterone as indicators to reflect alterations in morphology and hormone regulation in male *N. denticulata* after chlordane and lindane exposure.

2. Materials and methods

2.1. Shrimp collection and maintenance

Green neon shrimp (*N. denticulata*) were taken from rivers in Taipei County, northern Taiwan 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 7.4–7.8, dissolved oxygen concentration exceeded 7.3 mg/l, and hardness was 38–45 mg $CaCO_3$/l. Temperature was maintained at 25 ± 1 °C, and a 12-h light–dark photoperiod was used. Under these conditions, shrimp were fed twice a day and were allowed to acclimate for two weeks before testing.

2.2. Exposure of shrimp to 17β-estradiol, chlordane, and lindane

Ninety-six hour LC_{50} levels for chlordane and lindane for *N. denticulata* are 127.03 ng/l and 9.36 μg/l, respectively (Huang and Chen, 2004). Sublethal concentrations of chlordane and lindane were 1, 10 ng/l, and 0.1, 1 μg/l, respectively. Experiments with positive controls (10 and 100 μg/l 17β-estradiol in alcohol) with 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 150 male shrimp (body length, 13 ~ 16 mm) of the same size in each group (in a 10-l glass beaker, run in triplicate for each). Males were early identified as the endopod of the second pleopods has a masculine appendage (Fig. 1a) that is oval-shaped and is surrounded by cilia (Hung et al., 1993; Shy and Yu, 1998; Englund and Cai, 1999). Samples were taken at the end of days 1, 3, 7, 14, and 28.

2.3. Morphological study

In the morphological study, we observed the morphology of the masculine appendage after 3, 7, 14, and 28 days exposure. We also measured the length of the masculine appendage on the second pleopods (Fig. 1b) and the length of the cephalothorax. Data shown include the ratio of the lengths of the masculine appendage and cephalothorax.

2.4. Testing preparation

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.

2.5. Determination of hemolymph estradiol

Levels of estradiol were determined using an EIA kit (DSLabs, Webster, TX, USA). The assay uses the competitive binding enzyme immunoassay format (Maxey et al., 1992). In the assay, standards, controls, and unknowns containing estradiol are incubated with biotin-labeled estradiol and rabbit anti-estradiol anti-

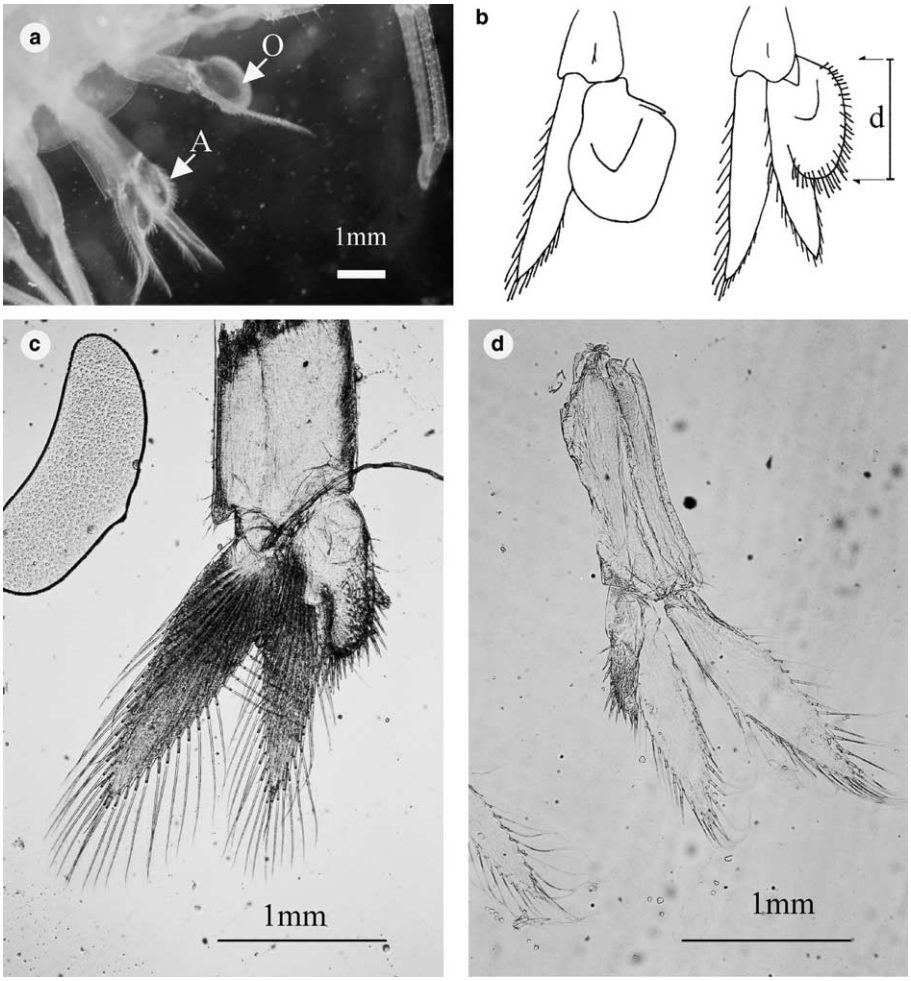

Fig. 1. The major characteristic of male *Neocaridina denticulata* in the control and treated group (100 μg/l 17β-estradiol). (a) Appearance of an oval-like endopod (O) on the first pleopod and masculine appendage. (A) Endopod of second pleopods with cilia (bar = 1 mm). (b) "*d*" indicates the length of the masculine appendage on the second pleopods. (c) Second pleopods of the control groups ($d = 0.9$ mm, cephalothorax = 5.38 mm), and (d) second pleopods of the treated group (100 μg/l 17β-estradiol) on day 7 ($d = 0.65$ mm, cephalothorax = 5.4 mm) (bar = 1 mm).

serum in microtitration wells where the unlabeled and biotin-labeled antigens compete for a limited number of anti-estradiol binding sites. After incubation and washing, the wells are incubated with streptavidin-HRPO, which binds to the biotinylated estradiol. The unbound streptavidin-HRPO is washed, followed by incubation with the substrate tetramethylbenzidine (TMB). An acidic stopping solution is then added, and the degree of enzymatic turnover of the substrate is determined by dual wavelength absorbance measurements at 450 and 620 nm.

2.6. Determination of hemolymph testosterone

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 anti-serum binding sites (Pradelles et al., 1985; Maxey et al., 1992). The product of this enzymatic reaction has a distinct yellow color and a strong peak absorbance at 405 nm by spectrophotometry.

2.7. Determination of hemolymph vitellogenin

The level of vitellogenin-like protein in the hemolymph was mediately determined by using an alkali-labile phosphate (ALP) measurement assay, and the quantity of ALP in the 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 of ice-cold 20% trichloroacetic acid (TCA) and incubated at room temperature for 15 min. The mixture was centrifuged at 10000×*g* for 10 min at 4 °C. The protein pellet was resuspended in 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 (Ellman et al., 1961; Gangé and Blaise, 2000).

2.8. Statistical analysis

Statistical analysis used Microcal™ origin 6.0. (Northampton, MA, USA, 1999). Experimental and control values were compared using Student's *t*-test (paired assay, $p < 0.05$).

3. Results

3.1. Levels of estradiol and testosterone in hemolymph

In our present study, high levels of estradiol were detected after males of *N. denticulata* treated with 17β-estradiol, chlordane, and lindane ($p < 0.01$) (Fig. 2). It was also interesting that the levels of testosterone in the hemolymph of 17β-estradiol-, chlordane-, and lindane-treated *N. denticulata* showed lower values compared with those of control shrimp, especially after 14 and 28 days of exposure ($p < 0.05$) (Fig. 3). The R^2 of standard curves in estradiol and testosterone assays were higher than 0.99; standard check and relative percent difference were less than 10% and 5%, respectively.

3.2. Vitellogenin levels in hemolymph

Treatment with 17β-estradiol, chlordane, and lindane changed the levels of vitellogenin (Vg) in the hemolymph, as determined by ALP assays. Vg levels in male *N. denticulata* hemolymph were induced by treatment with 100 µg/l 17β-estradiol on days 14 and 28, ($p < 0.05$), while 10 µg/l 17β-estradiol caused a significant induction of Vg levels only after 28 days of exposure ($p < 0.05$). Vg was induced in male *N. denticulata* treated with 10 ng/l chlordane for 14 and 28 days. There were no significant differences between treatment groups (0.1 and 1 µg/l lindane, and 1 ng/l chlordane) and the control group (Fig. 4). The R^2 of standard curve in vitellogenin assay was 0.9965 (>0.99); standard check

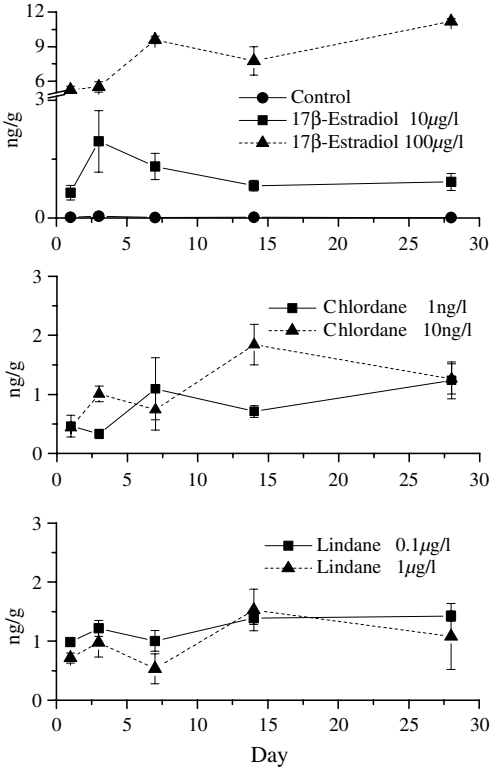

Fig. 2. Hemolymph estradiol response levels in male *Neocaridina denticulata* exposed to 17β-estradiol, chlordane, and lindane for 1, 3, 7, 14, and 28 days (mean ± SD, $n = 15$).

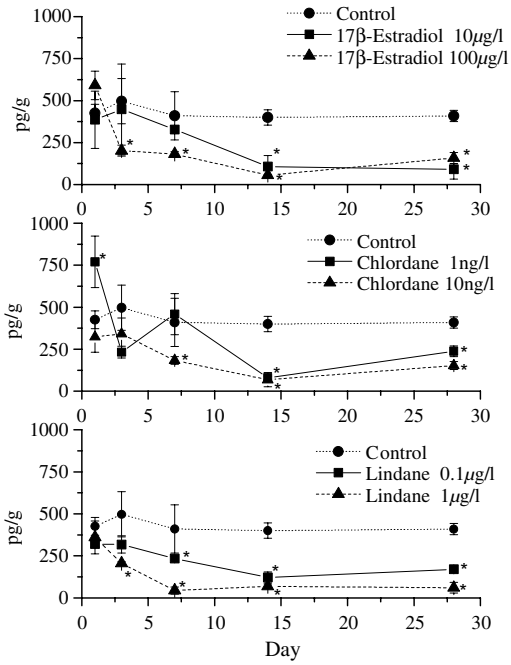

Fig. 3. Hemolymph testosterone response levels in male *Neocaridina denticulata* exposed to 17β-estradiol, chlordane, and lindane for 1, 3, 7, 14, and 28 days (mean ± SD, $n = 15$). An asterisk (*) indicates that the difference between the experimental and control group was significant at $p < 0.05$.

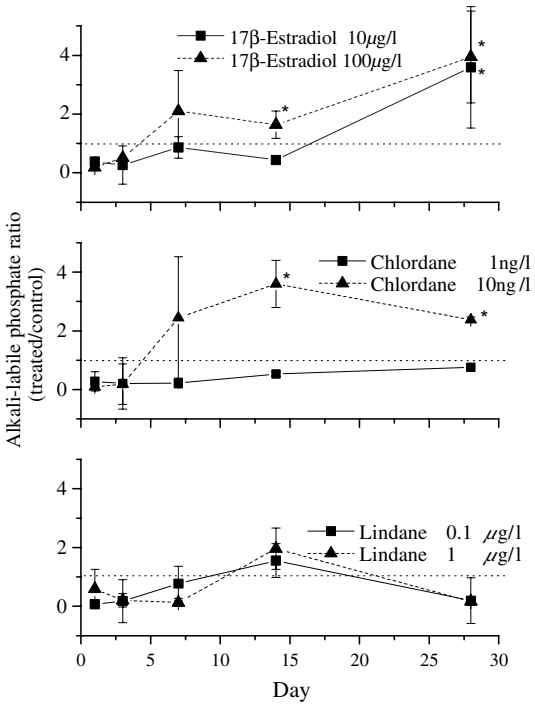

Fig. 4. Hemolymph APL response levels in male *Neocaridina denticulata* exposed to 17β-estradiol, chlordane, and lindane for 1, 3, 7, 14, and 28 days (mean ± SD, $n = 15$). An asterisk (✱) indicates that a difference between the experimental and control group was significant at $p < 0.05$.

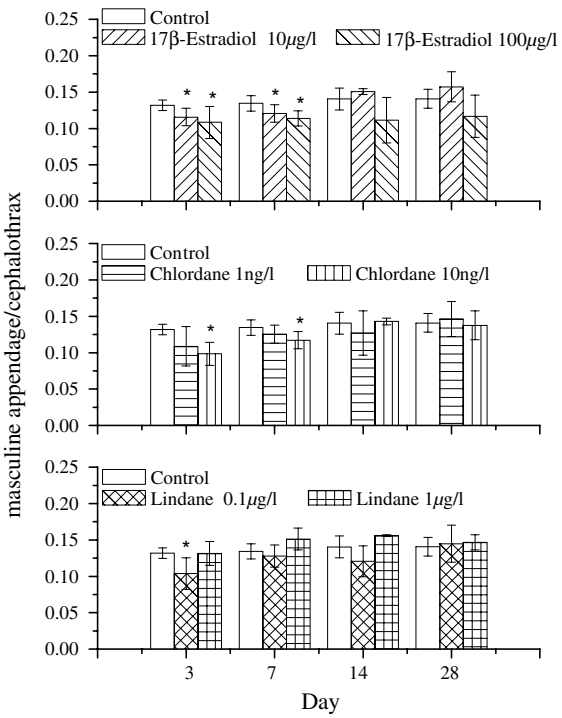

Fig. 5. Morphological influences on the masculine appendage in male *Neocaridina denticulata* exposed to 17β-estradiol, chlordane, and lindane for 3, 7, 14, and 28 days (mean ± SD, $n = 6$). An asterisk (✱) indicates that a difference between the experimental and control groups was significant at $p < 0.05$.

and relative percent difference were 2.11% (<10%) and 4.21% (<5%), respectively.

3.3. Morphological changes in the masculine appendage

Results of morphological studies showed that lengths of the masculine appendage from 17β-estradiol (10 and 100 μg/l)-treated shrimp were significantly shorter than those of control shrimp after 3 and 7 days of exposure ($p < 0.05$) (Figs. 1, 5). After exposure for 14 days, the lengths of the masculine appendage from 10 μg/l 17β-estradiol treated male shrimp had gradually increased, while those 100 μg/l 17β-estradiol treated individuals did not change. Furthermore, our results of exposure to OCPs showed very interesting patterns. After exposure to chlordane, changes in the length of the masculine appendage showed a similar pattern to that of 10 μg/l 17β-estradiol-treated shrimp. Although no significant difference was observed between lindane-treated shrimp and control individuals after exposure to 1 μg/l lindane, the trends of changes in the length of the masculine appendage revealed that exposure to 0.1 μg/l lindane caused shortening, while exposure to 1 μg/l lindane caused it to increase in length.

4. Discussion

Under normal conditions, testosterone is the major sex hormone in males, and only a very small amount of estrogen exists. However, estradiol can be found in male *N. denticulata* treated with high levels of 17β-estradiol, chlordane, and lindane (Fig. 2). Whether these substances can induce biological changes in male *N. denticulata* is a major concern. There is some evidence that exposure to EDCs can impact hormone regulation in organisms. It is reasonable to speculate that EDCs can cause reduction of testosterone if it is considered a xenoestrogen (Colborn et al., 1993; Parks and LeBlanc, 1996). We found that testosterone was significantly reduced with treatment using chlordane and lindane (Fig. 3). In other words, chlordane and lindane may possess a xenoestrogenic effect in *N. denticulata*.

We observed Vg synthesis in the chlordane-treated group of male *N. denticulate*, which implies that chlordane might have an estrogenic effect on this species (Fig. 4). A similar trend was found in lindane-treated groups although there were no statistically significant differences shown. This was probably due to the dose applied was insufficient to induce Vg synthesis, or was due

to the lack of an effect on the estrogen receptor of *N. denticulata* (Fig. 5). It has been shown that some Vg-like proteins found in crustaceans are used as precursors of vitellin (Vazquez Boucard et al., 2002). However, unlike fish, whose Vg synthesis occurs in the liver, synthesis has been observed in the hepatopancreas and ovaries in crustaceans (Tseng et al., 2001; Tsang et al., 2003). Under normal circumstances, a mature female organism usually shows a high level of estrogen, which can induce Vg synthesis, while a male or immature individual shows a much lower level of estrogen, which cannot trigger Vg synthesis. However, the circumstance changes when the male or immature organisms are exposed to a xenoestrogen. Vg synthesis can possibly be induced via the binding of the xenoestrogen to estrogen receptors (Kime et al., 1999).

Chlordane, lindane, and OCPs can cause estrogenic, anti-estrogenic, and anti-androgenic effects (Danzo, 1998). These effects may be caused by direct binding to estrogen and androgen receptors and the effect on the activities of sex hormone-metabolizing enzymes, such as DDT and DDE (Colborn et al., 1993; Danzo, 1998). Chlordane may reveal complex and variable biological functions as does estrogen (Cranmer et al., 1984; Cassidy et al., 1994). These include the findings of Vg synthesis, an increase in estrogen, and a reduction in testosterone on *N. denticulata*. Lindane has been shown to inhibit the cholesterol side-chain cleavage in mice, and may have both estrogenic (Lahiri et al., 1985) and anti-estrogenic effects (Chadwick et al., 1988; Cooper et al., 1989). Although lindane does not appear to directly alter the number and affinity of estrogen receptors (Laws et al., 1994), it might be able to compete with or affect the binding of estrogen to the receptors. Although Vg synthesis was not found in lindane-treated male *N. denticulata* in this study, the observed increased level of estrogen and decreased level of testosterone indicate that lindane may produce hormonal disorders in male *N. denticulata*.

Alteration in steroid hormone metabolism by EDCs can significantly affect steroid hormone-dependent processes, such as growth, reproduction, the sex ratio, morphology, and in some cases, a drop in the production of viable offspring (Colborn et al., 1993). Chlordane and lindane caused changes in hormone levels in male *N. denticulata*. Although we cannot definitely be certain whether such changes would handicap the reproduction of *N. denticulate*, the masculine appendage, a male sexual characteristic, was affected (Fig. 4). In some aquatic organisms, sexual characteristics are changed after exposure to EDCs (Colborn et al., 1993; Taylor and Harrison, 1999). For example, a reduction in the length of the phallus of male alligators was documented after exposure to OCPs (DDT, DDE, and DDD) in the Lake Apopka, FL, USA (Crain and Guillette, 1998; Taylor and Harrison, 1999). A similar intersexual result was observed in fish exposed to sewage effluent containing estrogenic activity (Denton et al., 1985; Jobling et al., 1996; Harshbarger et al., 2000). *Nucella lapillus* showed imposex with the development of a penis and vas deferens in females after exposure to tributyltin (TBT) (Gibbs et al., 1988). Studies on the biological effects of EDCs have made important contributions to elucidating some of the basic events in pathophysiology, which is a key element for risk assessment of EDCs (Lebel et al., 1998; Mantovani et al., 1999; van Wezel et al., 2000). In this study, it was obvious that the masculine appendage had changed after 3 and 7 days of exposure to 17β-estradiol, chlordane, and lindane. Furthermore, chlordane and lindane caused alterations in the structure of male characteristics of *N. denticulata* within a shorter time than in the other organisms mentioned above. Using the structure of the masculine appendage as an element in risk assessment is a simple and time-saving task. However, its reference requires further evaluation.

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