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# Synthetic Estrogenic Agents Do Not Interfere with Sex Differentiation but Do Inhibit Molting of the Cladoceran *Daphnia magna*

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Several anthropogenic chemicals not specifically designed to possess hormonal activity, such as some pesticides and plasticizers, exhibit estrogen-like activities in vertebrates. This is because these agents are able to bind to the estrogen receptor (McLachlan and Arnold 1996). There is substantial evidence that these agents are capable of interfering with male development in wildlife populations, including alligators (Guillette et al. 1994), turtles (Bergeron et al. 1994) and panthers (Facemire 1995).

Because of the role of cladocerans as an important link in food webs of freshwater ecosystems, whether hormonally active xenobiotics can also inflict adverse effects upon the endocrine processes in cladocerans is of concern. On the basis of the historical data on changes in the *Daphnia* sex ratio in Lake Mendota, Wisconsin, Dodson and Hanazato (1995) speculated that the decline in the proportion of males over several years may have been caused by some of the same estrogenic xenobiotics capable of perturbing male development in vertebrates.

Molting in arthropods is controlled by steroid molting hormones called ecdysteroids (Chang et al. 1993; Lachaise et al. 1993). In malacostracan crustaceans sexual differentiation of male crustaceans is controlled by the androgenic gland hormone alone (Charniaux-Cotton 1954) which is a protein (Hasegawa et al. 1993). Females have no androgenic glands, which accounts for their having differentiated into females. It is possible that a certain degree of structural overlap exists between the steroid molting hormones and estrogen-mimicking xenobiotics that would allow these mimics to bind to the ecdysteroid receptor and thus interfere with the molting process. In the present investigation we used the estrogenic pesticide endosulfan and a potent synthetic estrogen, diethylstilbestrol (DES) to determine whether exposure to these agents can affect male differentiation, as they do among vertebrates, and molting in *Daphnia magna,* an ideal representative cladoceran.

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## MATERIALS AND METHODS

*Daphnia magna* were purchased from the Carolina Biological Supply Company (Burlington, NC). A laboratory stock was cloned by raising a single parthenogenic female in artificial pond water at a temperature of 20 ± 1 °C, and pH 7.0-7.2. The medium for culturing the daphnids was that of Hebert and Crease (1980) with slight modification, $CaCl_2$ being used instead of $CaSO_4$. Artificial pond water was made by dissolving 48 mg $NaHCO_3$, 33 mg $CaCl_2$Ž2H$_2$O, 60 mg $MgSO_4$Ž7H$_2$O, and 0.5 mg KCl in 1 L of distilled water. Animals were fed a dried algae mixture (Carolina Biological Supply Company).

Endosulfan (99.9%) was purchased from Axact Standards Inc. (Commack, NY). Diethylstilbestrol (DES) was obtained from the Sigma Chemical Co. (St. Louis, MO). Stock solutions were prepared by first dissolving the chemicals in acetone immediately prior to each experiment.

Endosulfan acute toxicity data for *Daphnia magna* were already available (Fernandez-Casalderrey et al. 1994). Only acute toxicity of DES was tested. Four test concentrations plus a control containing 314 mg/L acetone were used. For each test concentration and control, 10 neonates (age < 24 hr) were introduced into a 30-mL beaker containing 25 mL test medium. The water was the same artificial pond water as described above. The daphnids were not fed and the medium was not renewed during the bioassay. The mortality was recorded at 48 hr. Control survival was always 100%. The 48-hr $LC_{50}$ with 95% confidence interval was calculated according to Gad and Weil (1984). DES was found to have a 48-hr $LC_{50}$ value of 1.09 mg/L and a 95% confidence interval ranging from 0.21-5.83 mg/L.

In the experiment on the possible effects of endosulfan and DES on male differentiation three concentrations of each chemical were used; 0.05, 0.10 and 0.15 mg/L endosulfan and 0.05, 0.10 and 0.20 mg/L DES. The selection of endosulfan concentrations was based on the no observed effects concentrations (NOEC) obtained by Fernandez-Casalderrey et al. (1994). For rates of filtration and ingestion, the NOEC value of endosulfan is 0.15 mg/L for *Daphnia magna*. The three DES concentrations used were approximately 1/20 - 1/5 of the 1.09 mg/L 48 hr-$LC_{50}$ value. Ten female neonates (age < 24 hr) for each concentration of each chemical and 20 female neonates (age < 24 hr) for the control were introduced into a 30-mL beaker filled with 25 mL test medium. Because acetone was used as a carrier, the control medium contained 15.7 mg/L acetone. The daphnids were fed the dried algae mixture at a concentration of 30 mg/L every three days and the test medium was replaced every six days. The test vessels were kept in a water bath at 20 ± 1 °C and under a light regime of 8 hr light/16 hr dark. These experimental conditions were selected because according to Kleiven et al. (1992) induction of sexual reproduction in *Daphnia magna* requires the simultaneous actions of a short daily photoperiod, food limitation and crowding.

Table 1. Proportions of males and male-containing broods produced by *Daphnia magna* treated with endosulfan and DES. Statistical analysis of the exposure groups and the control showed no significant differences ($P > 0.05$).

| Treatment | Number of adult daphnids | Total neonates | Proportion of males | Total broods | Proportion of male-containing broods |
|---|---|---|---|---|---|
| Control | 17 | 117 | 6.84% | 36 | 16.67% |
| Endosulfan (mg/L) | | | | | |
| **0.05** | 6 | 58 | 5.17% | 13 | 7.69% |
| 0.10 | 8 | 53 | 11.32% | 17 | 11.76% |
| 0.15 | 7 | 32 | 15.63% | 16 | 18.75% |
| DES (mg/L) | | | | | |
| 0.05 | 6 | 69 | 10.14% | 15 | 33.33% |
| 0.10 | 5 | 56 | 10.71% | 14 | 21.43% |
| 0.20 | 7 | 61 | 8.20% | 18 | 16.67% |

When a daphnid deposited eggs into its brood chamber, it was transferred individually into a 10-mL test tube filled with 8 mL test medium. The animals continued to be fed every three days and the medium was renewed every six days. Neonates, if any, were collected daily. The neonates were removed using a pipet and their sex was determined with the aid of a dissecting microscope. Mortality before reaching maturity was 15% for the controls and 20 - 50% for the exposure groups. The experiments were terminated after 40 days.

In the experiment to determine the possible effects of endosulfan and DES on molting the same concentrations of each chemical were used as in the Male Differentiation experiment. Ten first instar neonates (age < 12 hr) were placed individually in a 10-mL test tube containing 8 mL test medium for each concentration of each chemical and the control. All the experimental conditions were the same as described above except that the light regime was changed to 12 hr light/12 hr dark. The incidence of molting among all the specimens, regardless of their sex, was checked every 12 hr and the shed exoskeletons were carefully

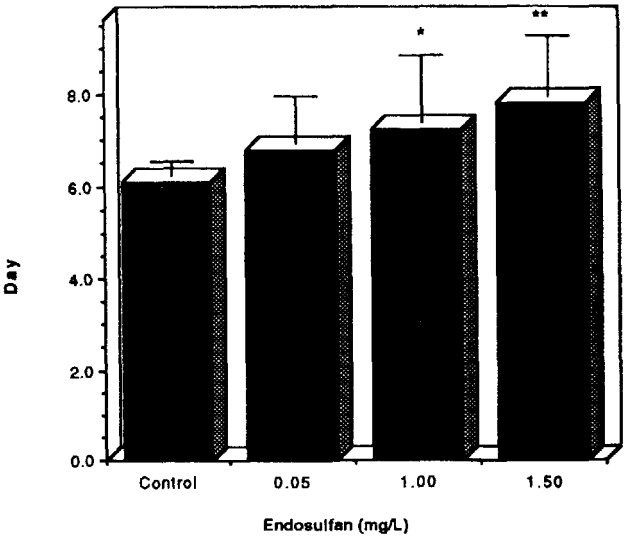


Figure 1. Effect of exposure to endosulfan on the molting of *Daphnia magna*. Error bars indicate the standard deviation. *P< 0.05, **P <0.01.

removed using a pipet. The experiments were ended after the daphnids reached the fourth instar. No mortality at any concentration occurred during the bioassays.

The hypothesis test as described by Daniel (1991) was used to test the differences between the proportions of males and between the proportions of broods containing males produced by exposed daphnids and the controls. One way analysis of variance (ANOVA) and Duncan's multiple comparison of group means (Tu 1985) were employed to test the difference between the time needed to finish four molts for the exposed daphnids and controls. The significance level was set at $P < 0.05$.

## RESULTS AND DISCUSSION

About two weeks after the initiation of each experiment, the *Daphnia magna* started to reproduce. The vast majority of clutches produced were unisexual. To calculate the proportions of male broods (Table 1), the numbers of broods consisting of males only and mixed broods were summed. That is, a mixed brood was considered as male for these calculations. Kleiven et al. (1992) in their study of sexual reproduction in *Daphnia magna* calculated proportions of male broods in the same manner. Table 1 shows the comparison of proportions of males and male broods produced by the daphnids treated with the various concentrations of endosulfan and DES, and by the control daphnids. No statistically significant differences were found between the proportions of males and between the proportions of broods containing males produced by the exposed daphnids and the control individuals at all concentrations of both compounds, $P > 0.05$ in all instances.

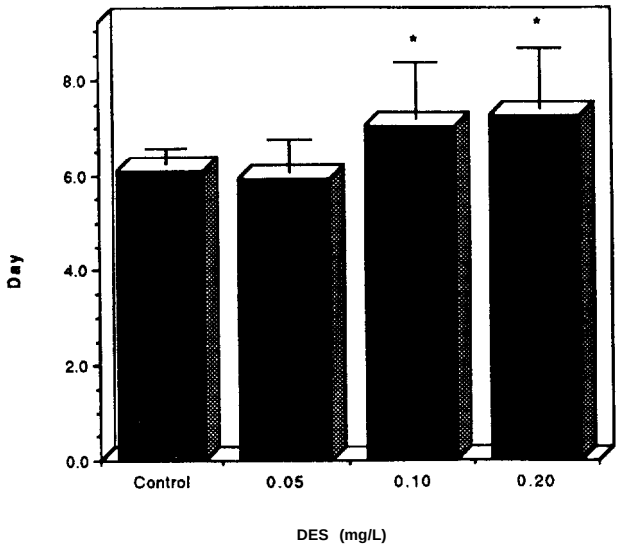


Figure 2. Effect of exposure to DES on the molting of *Daphnia magna.* Error bars indicate the standard deviation. *P <0.05.

Figures 1 and 2 show the effects of exposure to endosulfan and DES on the molting of *Daphnia magna* respectively. It took a significantly longer time for the *Daphnia magna* exposed to endosulfan at 0.10 and 0.15 mg/L and DES at 0.10 and 0.20 mg/L to complete four molts than for the control daphnids.

In malacostracan crustaceans, the sex of an individual is not distinguishable at hatching. The sexual characteristics develop later with each successive juvenile molt. As mentioned above, sexual differentiation in malacostracan crustaceans is controlled by the presence or absence of the androgenic gland hormone. When this hormone is present, a male develops, but in its absence a female develops (Fingerman 1987). In cladocerans sexual differentiation occurs before hatching and the sex of the individual can easily be identified at hatching. For example, the male neonate of *Daphnia magna is* characterized by a pair of long antennules.

Unfavorable environmental conditions such as crowding, food shortage, and short day lengths can trigger the transition from asexual to sexual reproduction, with males being produced initially (Du 1987). Parthenogenic females deposit male-producing eggs into the brood pouch after receiving appropriate environmental cues. Very likely, there is a chemical messenger acting like a hormone that passes the environmental cues to the ovary and instructs the ovary to lay male-producing eggs. Exogenous agents that would interfere with male differentiation could be doing so by affecting the process whereby this postulated hormone-like messenger

passes the environmental cues to the ovary. The present study failed to provide any evidence that endosulfan and DES affect male differentiation in *Daphnia magna*. Our results do not support the suggestion of Dodson and Hanazato (1995) that estrogenic xenobiotics may have been the cause for the decline in the proportion of males in the *Daphnia* population in Lake Mendota, Wisconsin.

The molting process in arthropods is regulated by a multi-hormonal system, but as stated above is under immediate control by molt-promoting steroid hormones, called ecdysteroids (Chang et al 1993; Lachaise et al. 1993). Since the structural similarities between hormonally active xenobiotics and steroid hormones such as estrogen are the basis for the hormonal activity of these mimics, we hypothesize that the inhibitory effects rendered by endosulfan and DES on the molting of *Daphnia magna* are due to structural overlaps between these agents and the endogenous steroid molting hormones. These similarities may enable these mimics to bind to ecdysteroid receptors, but they then act as antagonists rather than as agonists of the ecdysteroids. These antagonists presumably block the receptors for extended periods, preventing endogenous steroid molting hormones from binding to and turning on the receptor, thereby slowing the molting process. However, it is possible that this inhibitory effect of endosulfan and DES on molting is a more general response to stressors, not directly involving ecdysteroids and their receptors. This possibility is currently being studied in this laboratory. Further investigations are needed to determine whether other known steroid mimicking, hormonally-active xenobiotics can also affect the molting process in crustaceans. Pharmacokinetic studies on the binding of hormonally active agents to the steroid molting hormone receptor should provide important information about the roles of these mimics at the cellular level. Molting in crustaceans gives promise of being a good biological model for assessing the hormone-like activity of various agents.

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REFERENCES

Bergeron JM, Crews D, McLachlan JA (1994) PCBs as environmental estrogens: Turtle sex determination as a biomarker of environmental contamination. Environ Health Perspect 102:780-781

Chang ES, Bruce MJ, Tamone SL (1993) Regulation of crustacean molting: A multi-hormone system. Am Zool 33:324-329

Charniaux-Cotton H (1954) Découvete chez un Crustacé Amphipode (Orchestia gammarella) d'une glande endocrine responsible de la differentiation des caractères sexuels primaires et secondaires mâles. CR Acad Sci Paris 239:780-782

Daniel WW (1991) Biostatistics: A foundation for analysis in the health sciences (5th ed). John Wiley & Sons, Inc.

Dodson SI, Hanazato T (1995) Commentary on effects of anthropogenic and natural organic chemicals on development, swimming behavior, and reproduction of *Daphnia,* a key member of aquatic ecosystems. Environ Health Perspect 103 (Suppl 4):7-11

Du N (1987) Crustacean biology. Scientific Press, Beijing, China

Facemire C (1995) Reproductive impairment in the Florida panther: Nature or nurture? Environ Health Perspect 103(Suppl 4):79-86

Fernandez-Casalderrey A, Fernando MD, Andreu-Moliner E (1994) Effect of sublethal concentrations of pesticides on the feeding behavior of *Daphnia magna.* Ecotoxicol Environ Safety 27:82-89

Fingerman M (1987) The endocrine mechanisms of crustaceans. J Crust Biol 7:1-24

Gad SC, Weil CS (1984) Statistics for toxicologists. In: Hayes AW (ed) Principles and Methods of Toxicology. Raven Press, New York, pp 273

Guillette LJ, Gross TS, Masson GR, Matter JM, Percival HF, Woodward AR (1994) Developmental abnormalities of the gonadal and abnormal sex hormone concentrations in juvenile alligators from contaminated and control lakes in Florida, Environ Health Perspect 102:680-688

Hasegawa Y, Hirose E, Katakura Y (1993) Hormonal control of sexual differentiation and reproduction in Crustacea. Am Zool 33:403-411

Hebert PDN, Crease TJ (1980) Clonal coexistence in *Daphnia pulex* (Leydig): Another planktonic paradox. Science 207:1363-1365

Kleiven OT, Larsson P, Hobaek A (1992) Sexual reproduction in *Daphnia magna* requires three stimuli. Oikos 65:197-206

Lachaise F, LeRoux A, Hubert M, Lafont R (1993) The molting gland of crustaceans: Localization, activity, and endocrine control. J Crust Biol 13,198-234

McLachlan JA, Arnold SE (1996) Environmental estrogens. Amer Sci 84:452-461

Tu YQ (1985) Biostatistics. Higher Education Press, Beijing