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Evaluation of Selected Endocrine Disrupting Compounds on Sex Determination in *Daphnia magna* Using Reduced Photoperiod and Different Feeding Rates

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Many anthropogenic chemicals, such as organochlorine pesticides and plasticizers, have been shown to exert toxicity in vertebrates and invertebrates through disruption of the endocrine system (LeBlanc 1998). These effects include impairment of the reproductive and molting processes and interference with sexual development in offspring. From a mechanistic standpoint, endocrine-modulating compounds may have several sites of action such as binding to hormone receptors or alterations in steroid hormone metabolism (Baldwin et al. 1997; Oberdörster 1998).

Cladocerans (water fleas of the genus Daphnia) are important invertebrate species in aquatic ecosystems. They are dominant herbivores and an important food source to larval and juvenile fish. Adequate survival, growth and reproduction of daphnids are crucial for success in the environment, but more important is the ability of the species to survive adverse environmental conditions during winter. Most daphnids are cyclic parthenogenetic species capable of both asexual and sexual reproduction (Dodson and Frey 1991). The survival strategy in sexual reproduction is to produce sufficient numbers of resting or winter eggs known as ephippia. Resting eggs are haploid and require the presence of males for fertilization and development. These eggs then enter a diapause stage and hatch in spring as parthenogenetic females.

In *Daphnia magna*, the switch to male offspring production and subsequent sexual reproduction appears to be triggered by two classes of environmental cues; phenological (i.e., reduced photoperiod) and density dependence (Hobæk and Larsson 1990). The timing of sexual reproduction and the optimal ratio of male to female offspring appear critical for adequate ephippia production. Although growth and molting in crustaceans is regulated by a variety of hormones (Chang 1989), little is known about the biochemical mechanisms involved in daphnid sex determination and development. Estrogens and testosterone have been detected in *Daphnia magna* (LeBlanc 1998), but their physiological role remains unclear. In the present study, the influence of selected endocrine disrupting compounds on sex determination in *Daphnia magna* was investigated.

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MATERIALS AND METHODS

Daphnia magna Straus cultures (clone 5 obtained from Academy of Natural Sciences of Philadelphia) were individually held in 150-mL glass beakers containing 50 mL of H-H COMBO medium (Baer and Goulden 1998). All exposures were performed in an environmental chamber with constant temperature (20 ± 2 °C) and 8-hr light: 16-hr dark photoperiod (illumination ranged between 318 and 460 lx). This reduced photoperiod has been shown to cause male production in *Daphnia magna* (Hobæk and Larsson 1990). The medium was renewed and daphnids were fed a green algae species, *Ankistrodesmus falcatus,* at concentrations of 50,000 cells/ml (low food rate) or 150,000 cells/ml (high food rate) three times weekly (i.e., Monday, Wednesday and Friday).

Tributyltin chloride (TBT; 97%) and 4-nonylphenol (NP; ~85% based on p-isomers) were purchased from Fluka Chemika (Milwaukee, WI). Methoxychlor (MC; 95%), 1,1-bis (p-chlorophenyl)-2,2,2-trichloroethane (DDT) and 17β-estradiol (E_2; 98%) were purchased from Sigma Chemical Co. (St. Louis, MO). Stock solutions were prepared by dissolving the appropriate amount of chemical in acetone (HPLC grade). The total amount of acetone added to test solutions and controls was 0.1 mL/L.

To evaluate the influence of selected compounds on *Daphnia magna* sex determination, randomly selected neonates from laboratory cultures (< 24 hr old, 1 third brood, 1 daphnid/beaker) were used. Sublethal concentrations of NP (25 µg/L), MC (1.0 µg/L) and DDT (1.0 µg/L) were used in the high food study, and E_2 (270 µg/L) and TBT (1.0 µg/L) were added to the exposure regime along with the previous compounds in the low food study. All exposure concentrations were based on the no observed effects concentration (21-d NOEC) obtained by other investigators (Baldwin et al. 1997; Oberdörster et al. 1998) and confirmed by preliminary laboratory experiments (results not shown). During all exposures, temperature, dissolved oxygen, pH, total hardness, total alkalinity and conductivity were monitored weekly. All measurements were within acceptable limits: temperature, 21.6 ± 0.4°C; pH, 7.7 ± 0.02; total hardness (mg/L as $CaCO_3$), 112 ± 2.0, total alkalinity (mg/L as $CaCO_3$), 67 ± 2.7; conductivity (µmhos), 430 ± 8.0. Endpoints included adult survival, first day of reproduction, total number of broods (male and female), number of molts (low food only) and total number of live and dead neonates produced (male and female). The sex of neonates was determined after immersion in hot water (~60°C) for 30 sec and macroscopic observation using a dissecting scope. Males were identified by the presence of large, prominent first antennules.

Statistical comparisons between exposure groups and feeding rates were accomplished using one way analysis of variance (ANOVA) and either Bonferroni's T-test, Dunnett's or Fisher's least significant difference test for comparisons of exposure groups and control. Additional nonparametric tests were employed when the data was heterogeneous (Bartlett's test) and/or not normally

distributed (Shapiro-Wilk's). These tests included Steel's Many-One Rank test for equal replicates or Wilcoxon Rank Sum test for unequal replicates. Tests were performed using standard software (StatView®, SAS® Institute, Inc.).

RESULTS AND DISCUSSION

The environmental cue employed to stimulate male production was a reduced photoperiod of 8-hr light/16-hr dark. Under these conditions, *Daphnia magna* has been shown to produce an optimum male/female ratio of approximately 1:2, independent of different concentrations of *Scenedesmus acutus* (2 and 4 mg/L, dry mass) (Hobæk and Larsson 1990). An increase in male production is usually not seen until food restriction reaches starvation levels. In the present study, low and high food concentrations of 50,000 and 150,000 cells/ml of *Ankistrodesmus* respectively, were used. These levels correspond to approximately 2 and 6 mg/L, dry mass, respectively and both feeding rates were well above the threshold level for reproduction. However, differences in male production between the two feeding rates were observed (Tables 1 and 2). For example, statistically significant differences in % male broods occurred between control groups (7.0 and 22.3% for 150,000 and 50,000 cells/ml, respectively). In addition, the ratio of females/brood per males/brood in controls in the high food rate was 12.5 compared to 5.0 in controls fed the low food rate, although the variability rendered this observation inconclusive. In agreement with the present study, Larsson (1991) observed an increase in male production in *Daphnia pulex* using low food concentrations (30,000 cells/ml of *Scenedesmus acutus*), reduced photoperiod and crowding conditions. These results emphasize the variability in response to stimuli for sex determination in daphnid species.

Differences in feeding rates also had a major influence on male production during exposure to DDT, MC and NP. In Figure 1, statistically significant differences between the two food concentrations and their respective exposure groups were observed in the ratio of females/brood per males/brood. For example, exposure to 1 µg/L DDT using the high food rate resulted in a ratio of 10.1 compared to a ratio of 1.9 using a low food rate. Similarly, exposure to 25 µg/L NP using the high food rate resulted in a ratio of 11.7 compared to 1.5 using a low food rate. And finally, exposure to 1 µg/L, MC resulted in a ratio of 15.6 using the high food rate compared to 2.0 using the low food rate. These results demonstrate the importance of feeding rates in daphnid sex determination during exposure to environmental endocrine disrupting compounds. While these results may reflect differences in bioavailability of organic compounds with increasing concentrations of live algae, different food concentrations alone can influence sex determination and possibly mask the effects of endocrine disruption.

Table 1 shows the effect of DDT, MC and NP on fecundity and sex determination using the high food rate. No statistically significant differences between exposure and control groups were observed for any parameter. Although there were slight increases in the % male broods, total number of males produced and total number

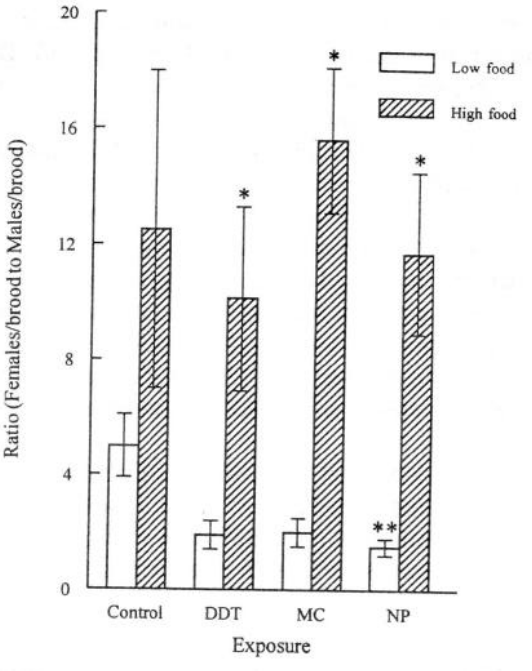

Figure 1. Comparison of the ratio of females/brood per males/brood using low and high food rates (50,000 and 150,000 cells/ml of *A. falcatus*, respectively). Values represent mean ± SEM. Exposure levels and sample size for controls, DDT, MC and NP are the same as presented in Tables 1 and 2. *Statistically significant from low food group ($p<0.05$); ** Statistically significant from control group ($p<0.05$).

of males produced per total brood in DDT and NP exposure groups, none were statistically significant.

Table 2 presents the influence of endocrine disrupting compounds on sex determination using a reduced food rate. Two additional compounds, E_2 and TBT, were employed in this study. The total number of molts between exposure groups and control was also measured in this study. Exposure with E_2 and TBT showed no statistically significant differences compared to control in any parameter. However, exposure to DDT, MC and NP resulted in an increase in the % male broods and a reduction in the total number of females as well as the total number of females/brood compared to controls. The ratio of females per brood/males per brood was lower for all DDT, MC and NP compared to controls, but only the NP group was statistically significantly different (control ratio of 5.0 compared with NP ratio of 1.5). The total number of molts was not statistically different in any exposure group compared to control except with MC.
The results from Table 2 are in contrast with those seen by other investigators. Zou and Fingerman (1997) evaluated diethylstilbestrol (DES) and endosulfan on sex differentiation and molting in *Daphnia magna*. No differences were seen in sex differentiation following exposure to these compounds but the duration of

time to produce four molts was statistically significantly longer following exposure to 1.0 and 1.5 mg/L endosulfan and 0.1 and 0.2 mg/L DES compared to controls. However, these investigators employed a dried algae mixture at a concentration of 30 mg/L compared to 2 mg/L dry mass of live *A. falcatus* in the present study. Baldwin et al. (1995) demonstrated that chronic exposure to 0.5 mg/L DES resulted in a decrease molting frequency in first generation daphnids but not in second generation daphnids. Molting was also not affected in adults. In another study, exposure of *Daphnia galeata mendotae* to high doses of NP (50 and 100 µg/L) slightly increased the production of female offspring while male production remained constant compared to controls (Shurin and Dodson 1997). The apparent discrepancies between these studies and the present study may be due to differences in species, food types and concentrations, and/or exposure levels of selected endocrine disrupting compounds.

Only a few studies are available that evaluate the influence of endocrine disrupting compounds on steroid metabolism in daphnids. NP has been shown to produce metabolic androgenization (i.e., inhibition of the metabolic elimination of conjugated metabolites of testosterone with concurrent increased levels of androgen within the organism) in *Daphnia magna* (Baldwin et al. 1997). An exposure concentration of 25 µg/L inhibited over 70% of the hydroxylated, sulfated and glucosylated testosterone conjugates while increasing the conversion of testosterone to relatively nonpolar reduced/hydrogenated products. Interestingly, metabolic androgenization was not observed following exposure of daphnids to 1.25 µg/L TBT (Oberdörster et al. 1998) or 0.5 mg/L DES (Baldwin et al. 1995). In the present study, increased male production was seen in NP but not in TBT and E_2 exposures. These results imply that endocrine disrupting compounds may alter sex determination in daphnids through alterations in steroid hormone metabolism. Further studies are needed to evaluate steroid hormone metabolism during sex determination following exposure to these compounds.

The present investigation, though based on comparatively small statistical sample, demonstrates that under certain environmental conditions (i.e., reduced photoperiod and low food concentrations) exposure to certain endocrine disrupting compounds pertubates sex determination of *Daphnia magna*. The alteration in the timing of sexual reproduction could severely impact the ability of the species to survive harsh environmental conditions. Premature appearance of males may ultimately lead to lower numbers of ephippia at a time when optimal production would be most needed. The subsequent decline or loss of daphnid populations could result in major detrimental consequences to aquatic ecosystems.

Table 1. The effect of DDT, methoxychlor (MC) and 4-nonylphenol (NE) on sex determination of *Daphnia magna* neonates using a reduced photoperiod (8-hr light/l6-hr dark) and high food rate (150,000 cells/ml of *Ankistrodesmus*) .

Exposure (µg/l)	Total # of Broods	% male Broods	Total # of Young	Total # of Females	Total # of Females/Brood	Total # of Males	Total # of Males/Brood	Ratio[a]
Control	7.3 ± 0.2	7.0 ± 2.3	218 ± 11	206 ± 13	28 ± 1.3	11 ± 6	1.6 ± 0.9	12.5 ± 5.5
DDT (1.0)	7.1 ± 0.2	14.0 ± 2.9	220 ± 7	194 ± 8	27 ± 0.9	26 ± 8	3.7 ± 1.0	10.1 ± 3.2
MC (1.0)	7.0 ± 0.0	9.8 ± 2.1	213 ± 5	203 ± 6	29 ± 0.9	10 ± 3	1.4 ± 0.4	15.6 ± 2.5
NP (25)	7.4 ± 0.2	15.8 ± 4.5	235 ± 11	203 ± 9	27 ± 1.3	31 ± 15	3.9 ± 1.7	11.7 ± 2.8

All values represent mean ± SEM. No statistically significant differences between exposure and control groups were observed ($p<0.05$). For all exposure groups n=10; except n=9 for NE. [a]Ratio of total number of females/brood to total number of males/brood.

Table 2. The effect of 17-β estradiol (E_2), tributyltin (TBT), DDT, methoxychlor (MC) and 4-nonylphenol (NP) on sex determination of *Daphnia magna* neonates using reduced photoperiod (8-hr light/l6-hr dark) and low food rate (50,000 cells/ml of *Akistrodesmus*).

Exposure (µg/l)	Total # of Broods	% male Broods	Total # of Young	Total # of Molts	Total # of Females	Total # of Females/Brood	Total # of Males	Total # of Males/Brood	Ratio"
Control	7.7 ± 0.3	22.3 ± 4.8	100 ± 15	9.7 ± 0.3	82 ± 12	11 ± 1.2	18 ± 4	2.3 ± 0.5	5.0 ± 1.1
E_2 (270)	7.0 ± 0.6	18.7 ± 3.3	84 ± 19	9.0 ± 0.6	67 ± 10	10 ± 0.6	16 ± 9	2.2 ± 1.1	6.8 ± 2.5
TBT (1.0)	7.8 ± 0.2	18.6 ± 3.5	112 ± 2	9.2 + 0.4	91 ± 5	12 ± 0.5	20 ± 5	2.7 ± 0.8	5.5 ± 1.3
DDT (1.0)	7.3 ± 0.4	39.3 ± 4.8[b]	79 ± 6	9.0 ± 0.0	48 ± 5[b]	7 ± 0.5[b]	31 ± 4	4.1 ± 0.6	1.9 ± 0.5
MC (1.0)	7.0 ± 0.0	40.0 ± 7.0[b]	74 ± 3	8.6 ± 0.2[b]	47 ± 4[b]	7 ± 0.5[b]	26 ± 3	3.8 ± 0.5	2.0 ± 0.5
NP (25)	7.4 ± 0.2	41.0 ± 4.6[b]	74 ± 9	9.2 + 0.4	43 ± 7[b]	6 ± 0.8[b]	31 ± 5	4.2 ± 0.5	1.5 ± 0.3[b]

All values represent mean ± SEM. For control and E_2, n=3; for DDT, n=6, for MC, NP, and TBT, n=5. [a]Ratio of total number of females/brood to total number of males/brood. [b]Statistically significantly different from controls at $p<0.05$.

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