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Effects of 4-Nonylphenol on Sexual Maturation in *Daphnia magna*

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The endocrine system of vertebrates and invertebrates has been shown to be sensitive to modulation by a variety of anthropogenic chemicals such as organochlorine insecticides and alkylphenols (LeBlanc 1998). Recently, 4-nonylphenol (NP), a major degradative product of nonylphenol ethoxylates, has drawn attention because of its intrinsic estrogenic potential. NP has been demonstrated to induce vitellogenin in male trout (Jobling et al. 1996), cause mouthpart deformities in chironomid larvae (Meregalli et al. 2001), and developmental retardation in *Daphnia magna* (LeBlanc et al. 2000).

D. magna are important invertebrate species in aquatic food webs. Most daphnids are cyclic parthenogenetic species capable of both asexual and sexual reproduction (Dodson and Frey 1991). Sexual reproduction is used to produce sufficient numbers of resting eggs known as ephippia. These eggs are haploid and require males for fertilization and development. As with most species, development of secondary sex characteristics is important for successful reproduction.

Recent studies have focused on evaluating the effects of endocrine-disrupting chemicals on the sexual maturation of male and female daphnids (Olmstead and LeBlanc 2000). Although daphnid sex is determined before birth, the development of male characteristics apparently involves additional hormonal processes during juvenile development and maturation (Mitchell 2001). Males are distinguished from females by differences in secondary abdominal processes, body length, rostrum, first antennules, breast margins, and first leg (Mitchell 2001). The development of secondary sex characteristics has been demonstrated to be altered by endocrine-disrupting chemicals. For example, diethylstilbestrol (DES) and methoprene stimulated development of the secondary abdominal process in female daphnids and androstenedione stimulated development of the first antennae in males (Olmstead and LeBlanc 2000). These endpoints may provide a sensitive monitor of endocrine disruption in these species. The goal of the present study was to investigate the influence of NP on sexual maturation in *D. magna*.

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MATERIALS AND METHODS

Daphnia magna Straus cultures (clone 5 from Academy of Natural Sciences of Philadelphia; 10/beaker) were held in 1 L glass beakers containing approximately 900 mL of H-H COMBO medium (Baer and Goulden 1998). The temperature was maintained at 20 ± 2° C and a 16-hr light:8-hr dark photoperiod (illumination ranged between 300 and 450 lux) was employed. The medium was renewed and daphnids fed a green algae species, *Ankistrodesmus falcatus* (7.5 x 10^6 algal cells/beaker) three times weekly (i.e., Monday, Wednesday, and Friday). These conditions allowed for continuous parthenogenetic reproduction in laboratory cultures. Males were produced by exposing the adults to 8-hr light:16-hr dark photoperiod, crowding conditions (25 daphnids/beaker), and low food (3.75 x 10^6 algal cells/beaker).

4-nonylphenol (NP; ~85% based on p-isomers) was purchased from Fluka Chemika (Milwaukee, WI). Stock solutions (3.6 mg/L) were prepared by dissolving 2 µL NP into distilled water followed by ultrasonication for 1 hr. In previous studies, recoveries of NP after preparation averaged 94% and recoveries after 48 and 72 hr ranged from 89 to 95% (results not presented).

To evaluate the effects of NP on sexual maturation of daphnids, randomly selected male and female neonates from laboratory cultures (<24 hr old, ≥ third brood) were used. Daphnids were exposed to a nominal test concentration of 100 µg/L NP (10 replicates containing 1 daphnid each) for 14 d. Daphnids were fed 7.5 x 10^6 algal cells/beaker at renewals three times weekly. Female daphnids were exposed under two photoperiods described as normal (16-hr light:8-hr dark) and reduced (8-hr light:16-hr dark). Endpoints for females during the exposure period included survival, total number of molts, total number of live neonates, and total number of deformed neonates. Males were exposed to NP under normal photoperiod. Endpoints for males during the exposure period included survival and the total number of molts. At the end of the 14-d exposure period, daphnids were placed on a glass microscope slide and medium was removed to immobilize the daphnids. Daphnids were observed for morphological alterations in carapace breast margins, head shape, and first leg (i.e., clasping hook for males). Total body length was measured from the top of the head to the base of the shell spine under 40x magnification using an ocular micrometer. The length of the secondary abdominal process in females and first antennae in males was also measured. The ratio of the abdominal process or first antennae per body length was calculated.

Statistical comparisons between exposure groups/conditions were accomplished using one way analysis of variance (ANOVA). Normality was determined using Shapiro Wilk test and homogeneity determined using Bartlett's test. The Tukey-Kramer Least Significant Difference adjusted t-test was used for comparisons between groups ($p<0.05$). All tests were performed using standard software (JMP IN®, SAS Institute, Inc., Cary, NC).

RESULTS AND DISCUSSION

The influence of photoperiod and NP on sexual maturation and reproduction in female daphnids is presented in Table 1. Reduced photoperiod had a stimulatory effect on the development of the secondary abdominal process in females. For example, the ratio of abdominal process/body length for daphnids under normal photoperiod averaged 0.182 compared to 0.203 for daphnids under reduced photoperiod. Other statistically significant differences influenced by photoperiod include delayed reproduction and a decrease in the total number of neonates produced in 14 d. Exposure to 100 µg/L NP for 14 d under normal photoperiod resulted in a statistically significant increase in the total number of molts, stimulation of the secondary abdominal process, and an increase in the total number of deformed neonates. However, under reduced photoperiod, no statistically significant differences were observed following NP exposure. No differences were observed in survival or other morphological characteristics at any experimental condition.

The influence of NP on development of the first antennae in males under normal photoperiod is presented in Table 2. NP caused a statistically significant decrease in the first antennae and the ratio of first antennae/body length. There were no significant differences in survival, the total number of molts, body length, or any other morphological characteristics in males following NP exposure under these conditions.

Little is known about the factors involved in normal development of male and female daphnid sexual characteristics. Mitchell (2001) observed that secondary sexual characteristics in males develop during the juvenile instars, possibly under a different hormonal process driving sex determination prior to birth. In this study, high temperature (30°C; photoperiod not reported) did not alter normal male development, but the rate was slightly faster due to a shorter instar period. High temperature also resulted in a higher incidence of intersex in the second generation daphnids with neonate females exhibiting male antennules. In the present study, the female abdominal process was stimulated under short photoperiod (20°C) as well as an increase in the first day of reproduction and a decrease in total neonate production. These conditions were similar to conditions that induced male production in later broods (Hobæk and Larsson 1990; Zhang and Baer 2000). The hormonal mechanisms involved in these observations under short photoperiod remain to be elucidated, but it is clear that environmental conditions such as photoperiod can influence the development of sexual characteristics in female daphnids. The development of male first antennae was not affected by short photoperiod (results not presented).

Other investigators have also demonstrated that the development of secondary sex characteristics can be altered during exposure to endocrine-disrupting chemicals. For example, DES and methoprene, a nonsteroidal estrogen and an insect juvenile growth hormone, respectively, stimulated the female abdominal process in *D. magna* but had no effect on the first antennae of males (Olmstead and LeBlanc 2000). Whereas the vertebrate androgen androstenedione stimulated development

Table 1. Influence of photoperiod and 4-nonylphenol (NP) on sexual maturation and reproduction in female daphnids.

Total # of Molts	Body Length (mm)	Abdominal Process (mm)	Ratio[e]	1st Day of Reproduction	Total # of Neonates	Total # of Deformed Neonates
(a) 16-hr light:8-hr dark						
7.1 ± 0.26^{b}	3.51 ± 0.016^{d}	$0.638 \pm 0.008^{b,c}$	$0.182 \pm 0.003^{b,c}$	$7.3 \pm 0.37^{c,d}$	$49 \pm 2.1^{c,d}$	0.0 ± 0.00^{b}
(b) 16-hr light:8-hr dark; 100 µg/L NP						
$8.1 \pm 0.23^{c,d}$	3.46 ± 0.021^{d}	0.719 ± 0.025^{a}	0.208 ± 0.007^{a}	$7.4 \pm 0.18^{c,d}$	42 ± 3.0	$2.3 \pm 0.53^{a,c,d}$
(c) 8-hr light:16-hr dark						
6.7 ± 0.30^{b}	3.55 ± 0.019	0.720 ± 0.013^{a}	0.203 ± 0.004^{a}	$8.8 \pm 0.21^{a,b}$	39 ± 1.4^{a}	0.0 ± 0.00^{b}
(d) 8-hr light:16-hr dark; 100 µg/L NP						
6.7 ± 0.37^{b}	$3.60 \pm 0.034^{a,b}$	0.693 ± 0.017	0.192 ± 0.005	$9.0 \pm 0.21^{a,b}$	35 ± 1.3^{a}	0.5 ± 0.22^{b}

Food levels were 7.5×10^{6} algal cells/beaker. All values represent mean ± SEM. [a,b,c,d]Indicates statistical significance compared to the specific treatment group/s (***a, b, c, d***)($p<0.05$). [e]Ratio of abdominal process/length.

of the first antennae of males with no effect on the abdominal process of females. All experiments in these studies were conducted under normal photoperiod (i.e.,

Table 2. Influence of 4-nonylphenol (NP) on sexual maturation in male daphnids.

Total # of Molts	Body Length (mm)	First Antennae (mm)	Ratio[a]
Control			
7.7 ± 0.33	2.71 ± 0.038	0.366 ± 0.020	0.133 ± 0.007
100 µg/L NP			
7.6 ± 0.24	2.73 ± 0.030	0.324 ± 0.000[b]	0.119 ± 0.002[b]

Food levels were 7.5 x 10^6 algal cells/beaker with 16-h light:8-hr dark photoperiod. All values represent mean ± SEM. [a]Ratio of first antennae/body length. [b]Statistically significant from control ($p<0.05$).16-hr light:8-hr dark).

In the present study, the actions of NP on the alteration of secondary sexual characteristics appear to be influenced by experimental conditions such as photoperiod. A slight NP-stimulatory effect on female molts and the abdominal process was observed only during normal photoperiod. NP exposure apparently mimicked the influence of short photoperiod in stimulating the development of the abdominal process. There was also an increase in the number of deformed neonates (curved or unextended shell spines and undeveloped second swimming antennae) observed in 5.5% of neonates following NP exposure. Interestingly, these NP effects were not observed under reduced photoperiod. NP increased body growth only under reduced photoperiod.

The role of NP in altering the development of secondary sex characteristics in daphnids remains to be clarified. In this respect, NP is acting similarly to DES and methoprene in stimulating development of the secondary abdominal process. However, NP had the opposite effect of androstenedione on the first antennae in males. This may not be too surprising since NP has been shown to have intrinsic estrogenic potential in vertebrates. For example, NP induced the formation of vitellogenin and inhibited testicular growth in male trout (Jobling et al. 1996). However, NP has also been shown to produce metabolic androgenization (i.e., inhibition of the metabolic elimination of conjugated metabolites of testosterone with concurrent increased levels of androgen) in *D. magna* (Baldwin et al. 1997). NP may modulate endocrine-dependent processes through several mechanisms of action. In view of these results, additional studies are needed to elucidate the hormonal mechanisms altered by NP exposure and interaction of NP with environmental conditions such as changing photoperiod.

The statistically significant increase in the total number of molts observed following NP exposure and normal photoperiod is difficult to explain. Molting in arthropods appears to be regulated, in part, by ecdysteroids (Chang et al. 1993). Exposure to chemicals with antiecdysteroidal activity, such as fenarimol, has been shown to decrease molting by lowering ecdysone levels (Mu and LeBlanc 2002). Molting has also been delayed following DES and endosulfan exposure (Zou and Fingerman 1997a). However, these effects may be due to a general stress response and not due to ecdysone antagonist activity. Previous studies with NP have not been shown to alter molting during several different experimental conditions (Baer and Owens 1999; Zou and Fingerman 1997b). Additional studies are needed to determine the influence, if any, of NP on molting during different experimental conditions, as well as the hormonal mechanisms involved.

Incorporating sexual characteristics into testing protocols to screen for endocrine-disrupting chemicals has been proposed (Olmstead and LeBlanc 2000). The results from the present study indicate that the development of secondary sex characteristics in daphnids can be altered following exposure to NP. However, experimental conditions may confound interpretation of reproductive and/or development of sexual characteristics following exposure to these endocrine-disrupting chemicals. Additional studies that standardize exposure conditions are warranted before inclusion of these endpoints into existing toxicity testing protocols.

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REFERENCES

Baer KN, Goulden CE (1998) Evaluation of a high-hardness COMBO medium and frozen algae for *Daphnia magna*. Ecotoxicol Environ Saf 39:201-206

Baer KN, Owens KD (1999) Evaluation of selected endocrine disrupting compounds on sex determination in *Daphnia magna* using reduced photoperiod and different feeding rates. Bull Environ Contam Toxicol 62:214-221

Baldwin WS, Graham SE, Shea D, LeBlanc GA (1997) Metabolic androgenization of female *Daphnia magna* by the xenoestrogen 4-nonylphenol. Environ Toxicol Chem 161:1905-1911

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

Dodson SI, Frey DG (1991) Cladocera and other Branchiopoda. In: Thorp JH, Covich AP (eds) Ecology and Classification of North American Freshwater Invertebrates, vol 1. Academic Press, Inc., San Diego, CA, p 723

Hobæk A, Larsson P (1990) Sex determination in *Daphnia magna*. Ecology 71:2255-2268

Jobling S, Sheahan D, Osborne JA, Matthiessen P, Sumpter JP (1996) Inhibition of testicular growth in rainbow trout (*Oncorhynchus mykiss*) exposed to estrogenic alkylphenolic chemicals. Environ Toxicol Chem 15:194-202

LeBlanc GA (1998) Steroid hormone-regulated processes in invertebrates and their susceptibility to environmental endocrine disruption. In: Guillette LJ (ed) Environmental Endocrine Disruptors: An Evolutionary Perspective. Taylor Francis, London, p 126

LeBlanc GA, Mu X, Rider CV (2000) Embryotoxicity of the alkylphenol degradation product 4-nonylphenol to the crustacean *Daphnia magna*. Environ Health Perspect 108:1133-1138

Meregalli G, Pluymers L, Ollevier F (2001) Induction of mouthpart deformities in *Chironomus riparius* larvae exposed to 4-n-nonylphenol. Environ Pollut 111:241-246

Mitchell SE (2001) Intersex male development in *Daphnia magna*. Hydrobiologia 442:145-156

Mu X, LeBlanc GA (2002) Environmental antiecdysteroids alter embryo development in the crustacean *Daphnia magna*. J. Exp Zool 292:287-292

Olmstead AW, LeBlanc GA (2000) Effects of endocrine-active chemicals on the development of sex characteristics of *Daphnia magna*. Environ Toxicol Chem 19:2107-2113

Zhang L, Baer KN (2000) The influence of feeding, photoperiod and selected solvents on the reproductive strategies of the water flea, *Daphnia magna*. Environ Pollut 110:425-430

Zou E, Fingerman M (1997a) Synthetic estrogenic agents do not interfere with sex differentiation but do inhibit molting of the cladoceran *Daphnia magna*. Bull Environ Contam Toxicol 58:596-602

Zou E, Fingerman M (1997b) Effects of estrogenic xenobiotics on molting of the water flea, *Daphnia magna*. Ecotoxicol Environ Saf 38:281-285