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Endocrine Disruptors in Developing Embryo on *Daphnia magna*

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Abstract : In crustaceans, as in other arthropods, the molt cycle and the physiological process of growth are controlled by molting hormones (MH) which are steroid hormones, the ecdysteroids. Ecdysteroids are major arthropod hormones which control both development (embryonic and larval molts, metamorphosis) and reproduction. The purpose of the present study was to evaluate both fenarimol and methoprene for embryotoxicity to daphnids. The embryotoxicity associated with each compound was assessed to discern whether the embryotoxicity of methoprene might be due to ecdysone agonist and the ecdysone antagonistic effects of fenarimol on Daphnia embryo. Exposure of daphnids for three weeks to 50 nM methoprene resulted in a significantly high incidence of offspring that exhibited general toxicity. This exposure concentration had significant effects on the overall number of embryo death. However, exposure to 3 or 1 μM fenarimol were no significant effects on the embryo toxicity. The incidence of both of these toxicity increased with methoprene exposure. This observation suggest that methoprene showed embryonic general toxicity during embryo development, while, only fenarimol showed weak general toxicity with early stages of embryonic development.

Keywords : endocrine disruptors, *Daphnia*, methoprene, fenarimol

Introduction

During the last two decades, a number of reports have appeared that have raised concerns about the development of reproductive problems in animal and man. There have been reports of alligators with abnormal male genital development and of reproductive changes in fish and birds. At the same time, there have been controversial reports of changes in human semen quality, alongside reports of an increasing incidence of congenital malformations of the male genital tract such as cryptochidism and hypospadias, and of an increasing incidence of testicular cancer[1].

Chemicals in the environment that mimic or block endogenous hormones might upset fine balance in ways that, while unexpected, are at least predictable based on the known biology of the endocrine system. The potential implications for human health as well as the health of numerous wildlife species are self-evidence[1,2].

The term "endocrine disrupting chemicals" is commonly used to describe environmental agents that alter the endocrine system. Collectively, chemicals with the potential to interfere with the function of endocrine systems are called endocrine disrupting chemicals (EDCs). EDCs have been defined as exogenous agents that interfere with the production, release, transport, metabolism, binding, action, or elimination of the natural hormones in the body responsible for the maintenance of homeostasis and regulation of developmental process[1,3].

The emerging field of scientific inquiry commonly referred to as "endocrine disruption" is thus of growing public health and perceived deleterious effects of environmental chemicals on the development or function of the reproductive system in species as diverse as snails, alligators, and humans.

Environmental endocrine disruption is a diverse group of industrial and agricultural chemicals in contact with humans and wildlife have the

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capacity to mimic or obstruct hormone function, not simply disrupting the endocrine system like foreign matter in a watchworks, but fooling it into accepting new instructions that distort the normal development of the organism[4].

The effects of environmental EDCs on fish populations have been documented worldwide. In the United Kingdom, the induction of hermaphroditism and estrogenic responses in rainbow trout and reach downstream of sewage treatment works in the United Kingdom at attributed to natural estrogens from humans, synthetic estrogens from birth control pills and chemicals with estrognic avtivity present in the effluent. In 1996, the US congress mandated that the US EPA (Environmental Protection Agency) develop and implement screening and testing methods for the evaluation of toxicity associated with endocrine-disrupting chemicals. In response, various teting approaches were considered that would serve to detect endocrine toxicity. A battery of testing methods has been in place for decades, for the evaluation of chemical toxicity as required under legislation such as the Federal Insecticide and Rodenticide Act and the Toxic Substancees Control Act. An expeditious means of evaluating the endocrine-disrupting potential of chemicals would be to incorporate endocrine-relevant endpoints into these existing testing protocols[4,5].

Arthropod (insects, crustaceans, and minor phyla) endocrinology is dominated by the involvement of terpenoid, ecdysteroid, and peptide hormones. Fragmented evidence suggests a role for vertebrate-type sex steroids (androgens, estrogens, progesterones) in regulating various reproductive processes in crustaceans[2].

Methoprene, [isopropyl (E,E)-11-methoxy-3,7,11-trimethyl-2,4-dodecadienoate] a juvenile hormone analogue, has been used for at least 20 years as the primary insecticide to control the pasture mosquito Aedes nigromaculis in Fresno County, California. First reports of apparent methoprene control failure were noted in a pasture west of Fresno in September 1998. Insufficient control was noted in 12 different pastures the following season from April to September 1999. In September of 1999, field trials were conducted to better ascertain the level of control. Results based on pupal counts from different methoprene formulations and rates of application indicated that in some pastures low levels of control were achieved with Altosid (Liquid Larvicide) and Altosid XR-G. Control with Altosid Pellets was reported at 52-99%[4].

This experiment was hypothesized that ecdysteroids also function in crustacean embryo development and accordingly ecdysteroid-modulating chemicals could adversely impact crustaceans by causing embryo toxicity. This experiment evaluated this hypothesis using *Daphnia magna*, a crustacean species that is commonly used in toxicity evaluations, and fenarimol (α-(2-chlorophenyl)β-(4-chlorophenyl)-5-pyrimdinemethanol) an agricultural fungicide that is known to inhibit cytochrome P450 enzymes and may accordingly interfere with ecdysone synthesis.

Materials and Methods

Daphnids

Daphnids were cultured and experimentally maintained in deionized water reconstituted with 192 mg/*l* $CaSO_4 \cdot H_2O$, 192 mg/*l* $NaHCO_3$, 120 mg/*l* $MgSO_4$, 8.0 mg/*l* KCl, 1.0 mg/*l* selenium, and 1.0 mg/*l* vitamin B12. Cultures were maintained at a density of 45 brood daphnids per liter of culture medium.

Culture medium was renewed and offsprings were discarded three times weekly. Brood daphnids were discarded after 3 weeks in culture and replaced with neonatal organisms. Cultured daphnids were fed twice daily with 1 m*l* (~4 mg dry weight) of Tetrafin^{tm} fish food suspenstion (Pet International, Chesterfill, New South Wales, Australia) and 2 m*l* (1.4×10^8 cells) of a suspension of the unicellular green algae, *Selenastrum capricornutum*. The algae were cultured in Bold's basal medium. The fish food suspension was as follows. 10 g of Tetranfin^{tm} in a weigh dish and add to 1 *l* of distilled water in blender. After blended the food on highest setting for 10 minutes, pour into 1 *l* beaker and cover with Parafilm^R. Leave in refrogerator overnight to settle solids, and pour suspended solution into food container and discard settled solid. Final solution should be approximately 4 mg/m*l* solid weight.

Culture and experimental solutions were maintained at 20°C under a 16 hour photoperiod.

These culture conditions maintained the daphnids in the parthenogenetic reproductive stage.

Embryo Exposure

Experiments were conducted to determine whether the embryo toxicity of the test compounds to daphnids was the result of direct exposure of embryo to the compounds in the brood chambers of the maternal organisms. Gravid daphnids were selected from cultures and examined microscopically for the level of development of embryos in the brood chamber. Embryos that were in early develpment (i.e., Stage 1) were removed by applying gentle pressure to the posterior region of the brood chamber with a dissecting needle. Extruded embryos were collected and pooled. Embryos were individually and randomly assigned to wells of 96-well microtiter plates along with 200 μl of media containing the desired concentration of test compound. Carrier solvent (ethanol) was present in all solutions at a concentration of 0.0005% (v/v). The number of embryos exposed to each treatment varied among experiments, depending upon embryo availability, and is indicated for individual experiments in the results.

Embryos were incubated at 20°C with a 16 hours photoperiod and were examined microscopically every 24 hours. Embryos were scored for stage of development and any abnormalities of development were recorded.

Toxicity Evaluations

Developmental stages used to score the embryos were previously described and depicted and summarizes as follows. Stage 1 : cleavage, embryo is symmetrically enclosed within two embryonic membranes with no evidence of cellular differentiation. Stage 2 : gastrulation, cellular organization and differentiation is evident, first embryonic membrane is ruptured as the embryo becomes asymmetrical. The blastopore can be discerned during Stage 2. Stage 3 : early embryonic maturation, the head capsule and second antennae are differentiated. Stage 4 : mid embryonic maturation, the eye becomes pigmented, second antennae remain confined by the second embryonic membrane. Stage 5 : late embryonic maturation, the second embryonic membrane has ruptured, freeing the second antennae. The shell spine remains curved along the anterior carapace edge. Stage 6 : fully developed nionate, setae are evident on the second antennae, the shell spine has fully extended from the carapace, and the organis is freely swimming. Treatment effects on the incidence of developmental abnormalities were statistically evaluated, comparing a single treatment to the control or ANOVA and Dunnett's t-Test.

Results and Discussions

Exposure of daphnids for three weeks to 50 nM methoprene resulted in a significantly high incidence of offspring that exhibited general toxicity. This exposure concentration had significant effects on the overall number of embryo death. However, exposure to 3 or 1 μM fenarimol were no significant effects on the embryo toxicity. The incidence of both of these toxicity increased with methoprene exposure. This observation suggest that methoprene showed embryonic general toxicity during embryo development, while, only fenarimol showed weak general toxicity with early stages of embryonic development. Fenarimol, an agricultural fungicide, that is known to inhibit cytochrome P450 enzymes and may accordingly interfere with ecdysone synthesis. The steroid biosynthesis inhibitor, ecdysone 20-monooxygenase, with an I50 of 10^{-6} M in disrupted glands, suggesting that it is a general P-450 inhibitor[6].

Exposure of Daphnia pulex to the insecticide

Table 1. Embryo Toxicity of Daphnids during 3 Days of Exposure to Fenarimol and/or Methoprene

chemicals/hours	0	24	48	60	84
vehicle control	24[3]	1[4]	1(2)[5]	0(2)	3(5)
fenarimol 3 μM	31	2	2(4)	0(4)	1(5)
fenarimol 1 μM	34	6	1(7)	0(7)	1(8)
F3 + M50[1]	34	7	5(12)	3(15)	1(16)
F1 + M50[2]	33	3	7(10)	3(13)	7(20)
Methoprene 50 nM	22	1	17(18)	1(19)	1(20)

[1]F3 + M50 : Fenarimol 3 μM + Methoprene 50 nM.
[2]F1 + M50 : Fenarimol 1 μM + Methoprene 50 nM.
[3]number of embryo at start.
[4]number of embryo death.
[5]( ) : total number of embryo death.

Table 2. Developmental Toxicity of Daphnids during 3 Days of Exposure to Fenarimol and/or Methoprene

chemicals/hours	0	24	48	60	84
vehicle control	43[3)]	0[4)]	2[c)]	2[d)]	2[f,g)]
Fenarimol 3 μM	43	1[a)]	10[c)]	11[d,e)]	26[f,g)]
Fenarimol 1 μM	43	1[a)]	4[c)]	7[d,e)]	15[f,g)]
F3 + M50[1)]	45	2[a)]	14[c)]	18[d,e)]	32[f,g)]
F1 + M50[2)]	45	2[a)]	11[c)]	14[d,e)]	21[f,g)]
Methoprene 50 nM	42	3[a,b)]	6[c)]	9[d,e)]	17[f,g)]

[1)]F3 + M50 : Fenarimol 3 μM + Methoprene 50 nM.
[2)]F1 + M50 : Fenarimol 1 μM + Methoprene 50 nM.
[3)]number of embryo at start.
[4)]number of embryo with abnormality.
[a)]irregular embryo membrane with pin-point perforations
[b)]prematured embryo.
[c)]ruptured embryo.
[d)]flared carapace, and a parts of internal contents were exposed.
[e)]undeveloped embryo, egg seemed to be in stage 1 or 2 but with eye spot.
[f)]undeveloped setae and flared carapace.
[g)]curved tailspine and undeveloped setae.

and juvenile hormone-mimic methoprene resulted in a decreased in the incidence of all-male broods and an increase in the incidence of all-female broods compared with controls. These effects were observed at nominal concentrations of 10 and 100 mg/*l*, within the upper range of concentrations at which methoprene is applied in the environment. Because methoprene has been found to bind to the mammalian retinoid X receptor. Juvenile hormone and ecdysteroids might play a role in the Daphnia sex determination system[7)].

Developmental abnormalities associated with methoprene exposure were not consistent with effects on early embryonic development as observed with fenarimol. Rather, developmental abnormalities consisted primarily of flared carapace or internal contents were exposed or attached to the plate wells. These observations suggest that methoprene interfered with latter stages of embryonic development, while only fenarimol didn't interfered embryonic development. The developmental abnormalities that occurred as a consequence of the ecdysteroid agonistic activity of methoprene, could significantly and adversely affect population abundance.

Species in the genus Daphnia are a major component of the fresh-water zooplankton throughout the world and for this reason have long been studied by limnologists. However, because of their ease of culture and short generation time, they have also attracted the attention of population ecologists. The maximum size reached by individuals of a particular species depends upon their food supply. And the head size depends directly on temperature, food supply, and amount of turbulence in the water. At a particular temperature, different species have a similar embryonic period ; at 10°C it is about 11 days, while at 25°C it is only about 2 days. Adults continue to moult throughout their life and as many as 28 moults have been recorded. It is known at low food concentrations Daphnia tend to live longer than at high concentration. Daphnia species reproduce either by cyclical or obligate parthenogenesis and populations ordinarily consist entirely of females. These individuals normally begin to reproduce after four or five juvenile instars and thereafter produce a new batch of eggs immediately after each moult[8)].

These results clearly demonstrated that ecdysteroids are critical to normal crustacean embryo development and environmental antiecdysteroids have the ability disrupt embryo development by interfering with development. Whether environmental chemicals with antiecdysteroid are shuffling of the gene pool and increasing genetic diversity in a population. Sexual reproduction includes many putative endocrine-regulated processes that are not evaluated endocrine activity have actually impacted crustacean populations remains to be established. The possibility rest in the degree to which this property is shared among environmental chemicals and the concentrations at which these chemical exist in the environment.

Sexual reproduction in daphnids is critical to population viability because fertilized eggs can sustain freezing or drying and thus provide a means of repopulation in new, more favorable environments or after environmental adversity. Sexual reproduction also provides a means for ren assessing toxicity only to parthenogenetically reproducting organisms. These include sex ratios of offspring, development of secondary sex

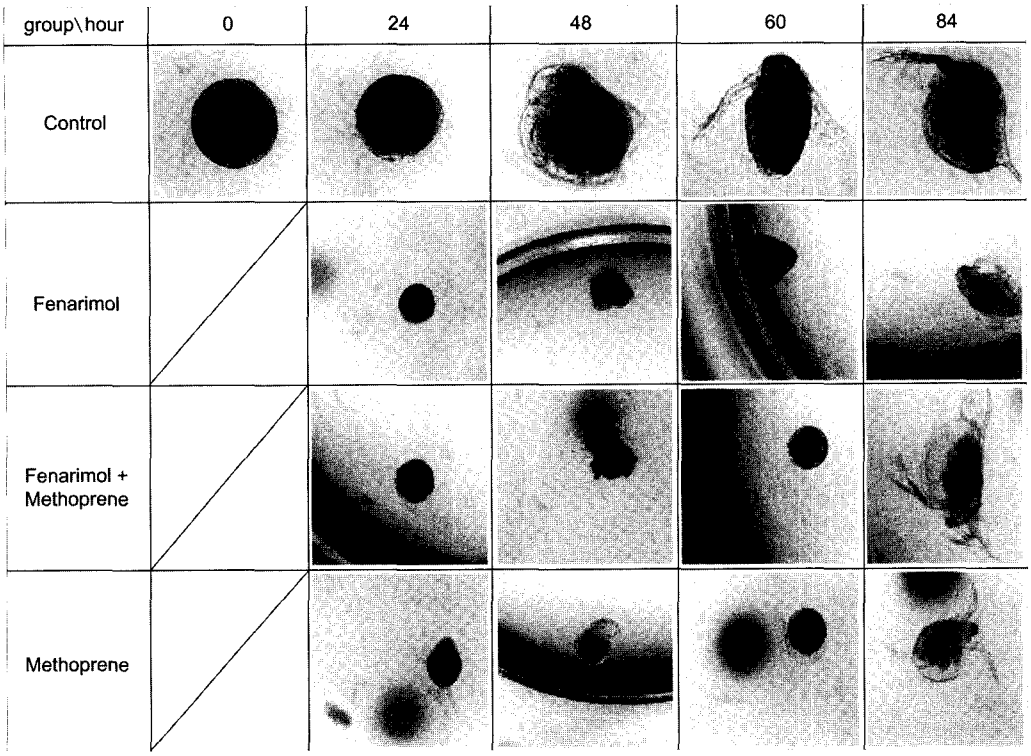

Fig. 1. Developmental toxicity of Daphnids during 3 days of exopsure to fenarimol and/or methoprene.

characteristics, fertility, and hatching success of resting eggs[9].

Embryo lethality has been observed during exposure of invertebrate embryos to some environmental chemicals. However, severe effects, such as lethality, may reflect toxicity that would impact any exposured life stage and not be indicative of specific developmental effects associated with the toxicant. Investigations into the toxicity of environmental chemicals such as the insecticide methoprene, alkylphenols, and the carbamate insecticide degradation product ethylenethiourea have revealed some effects on crustacean embryonic development at exposure concentrations less than those that are overtly toxic to neonatal organisms. These observed effects on the developing embryo also are suggesting of specific developmental toxiciy. For example, 4-nonylphenol and ethylenthiourea elicit specific, nonrecoverable abnormalities to the body form of daphnids as result of embryonic exposure. Exposure of daphnids to methoprene causes the development of grossly oversized or undersized embyos and embryos that are developmentally arrested during organogenesis[10].

Summary

In crustaceans, as in other arthropods, the molt cycle and the physiological process of growth are controlled by molting hormones (MH) which are steroid hormones, the ecdysteroids. Ecdysteroids are major arthropod hormones which control both development (embryonic and larval molts, metamorphosis) and reproduction. The purpose of the present study was to evaluate both fenarimol and methoprene for embryotoxicity to daphnids. The embryotoxicity associated with each compound was assessed to discern whether the embryotoxicity of methoprene might be due to ecdysone agonist and the ecdysone antagonistic effects of fenarimol on Daphnia embryo. Exposure of daphnids for three weeks to 50 nM methoprene resulted in a significantly high incidence of offspring that

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