Bull Environ Contam Toxicol (2008) 81:230–235
DOI 10.1007/s00128-008-9462-1

Elevated Concentrations of Ethinylestradiol, 17β-Estradiol, and Medroxyprogesterone have Little Effect on Reproduction and Survival of *Ceriodaphnia dubia*

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Received: 11 December 2007 / Accepted: 24 April 2008 / Published online: 18 July 2008

Abstract Wastewater effluent contains synthetic and natural hormones, often in complex mixtures, that may be associated with reproductive abnormalities in fish and other aquatic biota. We exposed the sentinel invertebrate *Ceriodaphnia dubia* to the natural estrogen 17β-estradiol (E_2), a synthetic estrogen, ethinylestradiol (EE_2), and a synthetic progestin, medroxyprogestone in a 7-day test. These compounds had no significant effect on reproduction or survival even at 10^6 times the concentrations at which reproductive effects have been documented in several fish species. *C. dubia* is routinely used for screening the toxicity of wastewater effluent. However, in the standard chronic 7-day exposure the endpoints of survival and reproduction were insensitive to several synthetic and natural vertebrate hormones. The *C. dubia* 7-day chronic toxicity test is probably not a useful monitoring tool for vertebrate hormones and their pharmaceutical analogs unless other sensitive endpoints such as maturation rates, molt frequency, and offspring sex ratios are incorporated in a practical manner.

Keywords Estrogen · Ethinylestradiol · Medroxyprogesterone · *Ceriodaphnia dubia* · Endocrine disruptor

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Currently, there is much concern over endocrine disrupting compounds that are found in sewage wastewater effluent and debate about the proper methods to screen and monitor these compounds. The presence of various estrogenic substances in wastewater is linked to reproductive abnormalities in exposed fish populations (Mills and Chichester 2005). Vertebrate hormones and their synthetic analogs have been detected in wastewater effluents in the ng/L range (Kolpin et al. 2002; Petrovic et al. 2002, 2004; Kolodziej et al. 2003), and laboratory and field evidence suggest that exogenous estrogen exposure can impair the reproductive health of various fish species (Lange et al. 2001; Jobling and Tyler 2003; Nash et al. 2004; Mills and Chichester 2005). Although greater attention has focused on estrogenic compounds (Petrovic et al. 2004), progestins, progesterone, androgens, and their synthetic analogs have also been detected in wastewater effluents in concentrations similar to those of estradiol (Kolpin et al. 2002; Kolodziej et al. 2003).

Invertebrate sentinel species that are commonly used in effluent monitoring may be useful screening tools for vertebrate hormones and their pharmaceutical analogs. *Ceriodaphnia dubia* is a freshwater cladoceran that is sensitive to many different types of contaminants and is often used for monitoring the chronic toxicity of many industrial and municipal effluents. It is an attractive monitoring species because of its sensitivity, filter feeding, and key position in many aquatic food webs. It also has a short life cycle, is easy

to culture, and is small, minimizing sample volumes needed for testing (Mount and Norberg 1984; USEPA 2002). A fast reproducing invertebrate model like *C. dubia*, with a short life cycle that is sensitive to vertebrate endocrine disruptors would be a valuable research and monitoring tool. To preserve biodiversity, it is also important to characterize the effects of these environmental contaminants in aquatic crustaceans as well as fish.

We tested the effects of ethinylestradiol (EE_2) and 17β-estradiol (E_2), two potent estrogens found in wastewater effluent, and medroxyprogesterone, a progestin recently detected in wastewater effluent, on reproduction and survival in *C. dubia*. EE_2 is a strong estrogen agonist that is used in oral contraception and has been detected in municipal wastewater, some streams in the United States (Desbrow et al. 1998; Kolpin et al. 2002; Kolodziej et al. 2003), and agricultural and aquaculture effluent (Kolodziej et al. 2004) in low ng/L concentrations. E_2 is a natural estrogen excreted by humans and other animals and is approximately 20 times less potent in inducing a vitellogenic response than EE_2 in some species of fish (Sumpter and Johnson 2005). Estrogens are typically found in wastewater at concentrations of 0.1–20 ng/L (Desbrow et al. 1998; Baronti et al. 2000; Kolpin et al. 2002; Kolodziej et al. 2003), and E_2 has been detected in concentrations as high as 93 ng/L (Kolpin et al. 2002) Environmentally relevant concentrations (1 ng/L^{-1} µg/L) can cause adverse reproductive effects in fish (Lange et al. 2001; Parrott and Blunt 2005). In various crustacean taxa, estrogens in the low µg/L to low mg/L range are reported to inhibit naupliar and larval development (Andersen et al. 2001), and alter vitellin levels (Ghekiere et al. 2006). Diethylstilbestrol, a potent estrogenic compound, alters molting frequency and fecundity in *Daphnia magna* at relatively high concentrations (0.5 mg/L) (Baldwin et al. 1995). All of these effects occur despite the fact that there is thought to be no estrogen receptor in crustaceans (Thornton et al. 2003).

Medroxyprogesterone is the progestin found in many human birth control pills and animal hormone treatments. It has been detected in US. wastewater in a range of concentrations from <0.4 to 14.9 ng/L (Kolodziej et al. 2003). The effects of medroxyprogesterone are of particular concern because some progestins are powerful preovulatory pheromones for fish at concentrations as low as 0.03 ng/L (Kolodziej et al. 2003). No data are available on the potential effects of medroxyprogesterone on *Daphnia* or other invertebrates. However, progesterone, 17β-hydroxyprogesterone, and 17α-hydroxyprogesterone can stimulate ovarian maturation and vitellogenesis in decapod crustaceans (Yano 1985; Quackenbush 1992; Zapata et al. 2003). Also, *D. magna* exposed to 100 µg/L progesterone from neonate to adulthood produced more male dominated broods, suggesting a possible effect of progestins on sexual development and sex determination (Kashian and Dodson 2004).

Using the USEPA's chronic *C. dubia* test, we examined both survival and reproduction of *C. dubia* exposed to a range of concentrations of E_2, EE_2, and medroxyprogesterone. We hypothesized that chronic exposure to these three hormones might disrupt *C. dubia* survival and reproduction at environmentally realistic concentrations.

Materials and Methods

Treatment concentrations in acute 48 h exposures of *C. dubia* neonates included 0.005, 0.05, 0.5, 1, 5 mg/L, a solvent control, and control for each EE_2, E_2, and medroxyprogesterone test. Chronic *C. dubia* exposure concentrations were chosen based on these 48 h tests. We were able to calculate an LC_{50} for EE_2 and used concentrations well below this value for the chronic exposures. Only the highest estradiol concentration had significant toxicity so chronic exposures were chosen below 5 mg/L. Medroxyprogesterone was not acutely toxic at any concentration used in the 48 h exposures, so the same concentrations were used for the chronic test as they exceeded maximal concentrations found in the environment.

EE_2, E_2, and medroxyprogesterone were purchased from Sigma Chemical Co. (St. Louis, MO, USA) and dissolved in reagent grade acetone to make 1,000 mg/L stock solutions. Treatment solutions were made daily by diluting stock solutions in reconstituted water used in culturing. All treatments, including the solvent control, received the same amount of solvent. The control treatment (non-solvent) received only reconstituted water.

Ceriodaphnia dubia survival and reproduction were tracked in a chronic 7-day continuous exposure, static renewal assay as described by the USEPA (USEPA 2002). *C. dubia* stock cultures and experimental organisms lived in moderately hard reconstituted water prepared according to USEPA guidelines using reagent grade salts and Type I water (Barnstead International) (USEPA 2002). Batches of water were kept in polyethylene carboys under continuous aeration and tested for total hardness, alkalinity, and pH. Hardness was maintained between 80 and 84 mg/L as $CaCO_3$, alkalinity between 60 and 68 mg/L as $CaCO_3$, pH 8.20–8.3. *C. dubia* were housed in a temperature controlled room under a 16:8 light dark cycle at 25°C. Neonates were placed in 15 mL of exposure solution in 30 mL polystyrene portion cups. Daphnids were maintained individually in cups and fed 0.1 mL *Selenastrum capricornatum* (3.0×10^7 cells/mL) and 0.1 mL YTC daily. Cups were rinsed with type I water prior to use and air-dried to remove any particulates; cups were not reused. Both *S. capricornatum* and YTC were purchased from Aquatic Biosystems (Ft. Collins, CO); YTC had total solids measured at 1.710 g/L. Each treatment was replicated 10 times and replicate cup locations were randomized.

Reproduction (the number of neonates produced) in chronic exposures was compared among treatments by one-way ANOVA. Chi-Squared tests were used to compare differences in mortality between treatments in chronic tests. Experimental data were analyzed using Systat 9.0 or JMP 6.0 software.

Results and Discussion

We did not find any significant differences in mortality during the chronic 7-day exposure of *C. dubia* to E_2, EE_2, or medroxyprogesterone for the concentrations chosen (Fig. 1). *C. dubia* mortality was low and not significantly different among treatments in any of the EE_2, E_2, or medroxyprogesterone tests.

No changes in *C. dubia* reproduction were detected in the EE_2 or medroxyprogesterone chronic 7-day tests, and in the E_2 test, only one treatment produced a slightly lower number of neonates than other treatments in the test (Fig. 2a–c). Neonate production in solvent control and control treatments was not significantly different in E_2, EE_2, and medroxyprogesterone experiments, and these two controls were pooled for further analyses. The number of neonates produced did not differ among treatments in the medroxyprogesterone ($F_{5,48} = 1.956$, $p = 0.1160$, Fig. 2c) or EE_2 ($F_{5,50} = 1.481$, $p = 0.362$, Fig. 2a) tests. Significant differences in number of offspring produced were found between treatments in the E_2 exposures ($F_{5,45} = 2.807$, $p = 0.036$). A post-hoc Tukey analysis showed that only the 0.05 mg/L E_2 was significantly lower than the control treatment ($\alpha = 0.05$) (Fig. 2b).

The synthetic and natural vertebrate hormones EE_2, E_2, and medroxyprogesterone had little effect on *C. dubia* mortality and reproduction even at concentrations much higher than those found in the environment. Only the 0.05 mg/L exposure in the E_2 exposures had significantly lower fecundity than controls (Fig. 1b). However, reproduction was not affected in a dose-dependent fashion in this or in any other estradiol treatment, making this finding difficult to interpret and perhaps spurious.

A great deal of recent research has focused on the effects of vertebrate steroids on invertebrates, including several recent review articles that focus on the role of steroid hormones in aquatic invertebrates in general (Kohler et al. 2007; Lafont and Mathieu 2007) and crustacean endocrine toxicology, including *D. magna* (LeBlanc 2007; Tatarazako and Oda 2007). The effects of vertebrate steroids and their synthetic analogs in cladocerans and copepods are most relevant to this study.

Several studies have shown that exogenous estrogens or antiandrogen exposure may affect maturation and alter the timing of molting in aquatic crustaceans. For example, nonylphenol, E_2, atrazine (A), benzo(a)pyrene, and

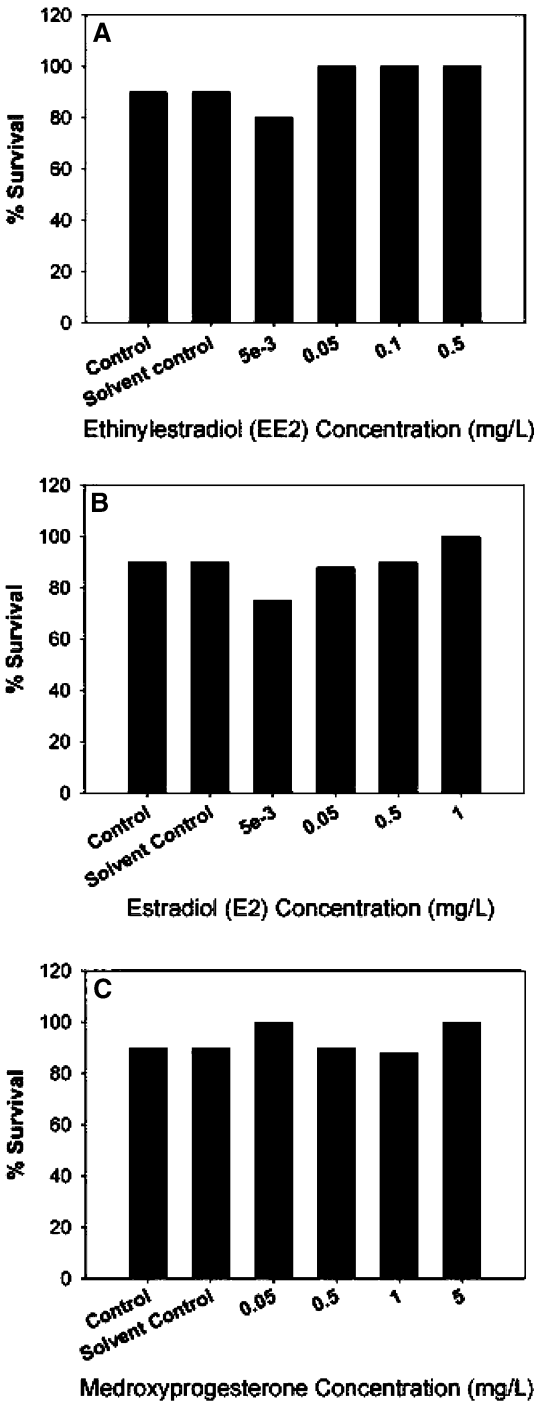

Fig. 1 *Ceriodaphnia dubia* survival during chronic 7-day exposure to (**a**) ethinylestradiol, (**b**) 17β-estradiol, and (**c**) medroxyprogesterone

di(ethyl-hexy-pathalate) (DEHP) did not affect fecundity and survival of the estuarine copepod *Eurytomora affinis*. However, in the same µg/L range, some of these compounds delayed or inhibited the transformation of nauplii to copepods. Binary combinations of E_2 + A, E_2 + NP, and E_2 + DEHP shifted the male:female ratio in the F1 generation so that more females were produced (Forget-Leray et al. 2005).

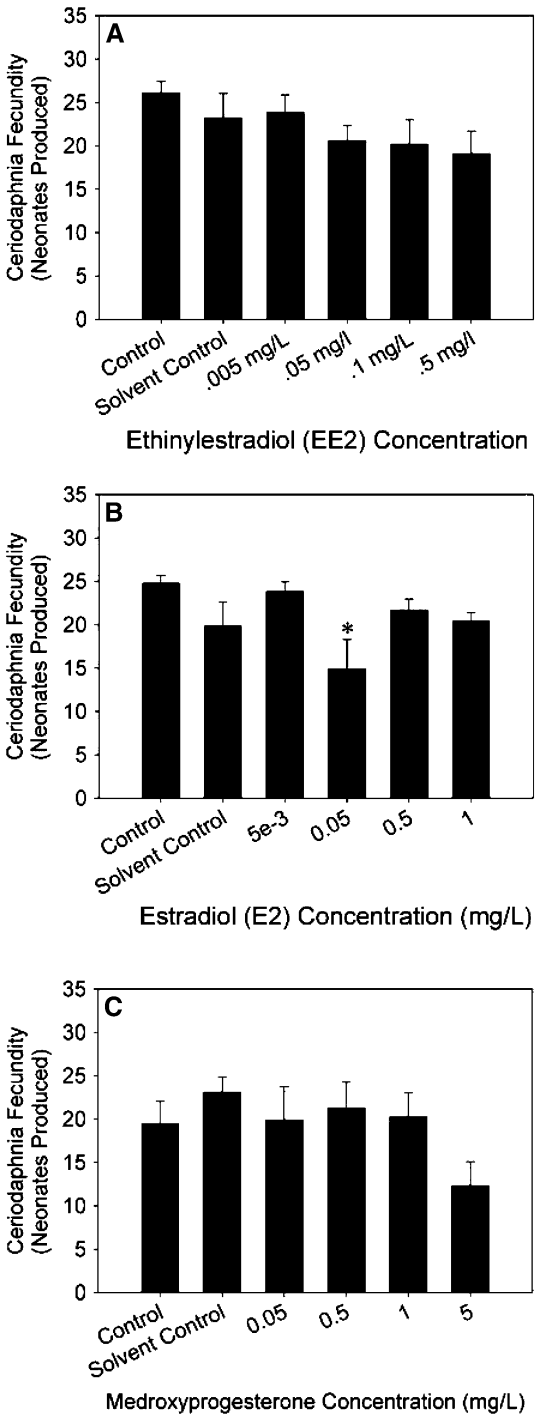

Fig. 2 *Ceriodaphnia dubia* neonate production during chronic 7-day exposure to (**a**) ethinylestradiol, (**b**) 17β-estradiol, and (**c**) medroxyprogesterone. An asterisk (*) indicates a significant difference compared to controls ($p < 0.05$)

Zou and Fingerman (1997a, b) tested a number of xenoestrogens and found that they do not affect sex differentiation in *D. magna*, but they inhibited molting in the low mg/L range. Diethylstilbestrol, Arochlor 1242, PCB 29, and diethyl phthalate alter the molt cycle, possibly by mimicking ecdysteroids. However, low mg/L concentrations are much greater than what is generally found in the environment (Zou and Fingerman 1997a, b). Also, there is little evidence supporting the idea that steroid estrogens such as EE_2 and E_2 have ecdysteroidal activity in *D. magna* (Tatarazako and Oda 2007) or cell based assays (Dinan et al. 2001). Other compounds that are estrogenically active in vertebrates such as 4-*tert*-octylphenol, bisphenol A, and *p*-nonylphenol did not show ecdysteroidal effects in *D. magna* (Tatarazako and Oda 2007).

Kashian and Dodson (2004) did not observe any effects of estradiol on *D. magna* sex determination, reproduction, growth, or survival at concentrations of 1, 10, and 100 µg/L (Kashian and Dodson 2004). In long term exposures to EE_2, *Sida crystallina* had a shortened juvenile phase at concentrations above 100 µg/L. Moreover, *Ceriodaphnia reticulata* had a higher mortality rate in juveniles at concentrations above 200 µg/L. Exposure of *S. crystallina* and *C. reticulata* to EE_2 did not cause changes in mortality of adult animals, birth rate, number of juveniles per female, or net reproduction rate (Jaser et al. 2003). In a multigenerational study of *Diaphanosoma celebensis*, a euryhaline cladoceran, exposure to 10–1,000 µg/L E_2 increased neonate production at a younger age relative to the controls in the parental and two subsequent generations. These effects were not observed in the third generation (Marcial and Hagiwara 2007).

Taken together, these studies show that particular estrogenic compounds may have some effect on maturation and development in several crustacean species in the mg/L and µg/L concentration range. However, most of the strongly estrogenic hormones (e.g. EE_2, E_2, estrone) that make up 80–90% of wastewater effluent estrogenicity are found in the environment in ng/L or sub ng/L concentrations (Desbrow et al. 1998). Thus it will be important to verify that these effects occur at environmentally relevant concentrations and that they cause population level changes in aquatic crustaceans. Recent mesocosm studies suggest that exposure to environmentally realistic concentrations of either EE_2 or NP reduces the abundance of cladocerans and copepods (Hense et al. 2005). It will also be important to understand the differences in sensitivity among different invertebrate species.

We did not find any effect of medroxyprogesterone on survival and reproduction in *C. dubia*. However, the fact that *D. magna* exposed to progesterone produced more male dominated broods, suggests that progesterone and progestins may possibly have ecdysteroid like effect (Kashian and Dodson 2004; Tatarazako and Oda 2007). Future studies with medroxyprogesterone should focus on other endpoints besides survival and reproduction such as molt frequency and male neonate production (Tatarazako and Oda 2007).

Ceriodaphnia dubia are often used as a sentinel species in monitoring wastewater effluent, thus it is important that survival and reproduction of this animal was not affected by

these hormones at mg/L concentrations. The *C. dubia* 7-day chronic toxicity test is probably not a useful monitoring tool for vertebrate hormones and their pharmaceutical analogs unless other sensitive endpoints such as maturation rates, molt frequency, and offspring sex ratios are incorporated in a practical manner.

Acknowledgments This research was funded by the Albert J. Ryan Foundation. The authors would like to thank Nayoung Yi and Brittany Crosby for their help in carrying out these experiments.

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