Ecotoxicology and Environmental Safety 72 (2009) 1545–1550

Contents lists available at ScienceDirect

Ecotoxicology and Environmental Safety

journal homepage: www.elsevier.com/locate/ecoenv

# Modulation of sex ratios in *Daphnia magna* following multigenerational exposure to sewage treatment plant effluents ☆

K.N. Baer *, M.D. McCoole, M.D. Overturf

*Department of Toxicology, College of Pharmacy, The University of Louisiana at Monroe, 700 University Avenue, Monroe, LA 71209, USA*

ARTICLE INFO

*Article history:*
Received 7 August 2008
Received in revised form
27 October 2008
Accepted 13 December 2008
Available online 3 February 2009

*Keywords:*
Sex ratios
*Daphnia magna*
Sewage treatment plant effluent
Multigenerational
Methyl farnesoate
STPs
MSTPs

ABSTRACT

The influence of sewage treatment plant effluents on sex ratios in *Daphnia magna* was investigated. Female daphnids were acclimated for several generations to effluents from a municipal sewage treatment plant and a residential oxidation lagoon and then placed under conditions to maximize male offspring production. Both effluents resulted in a statistically significant decrease in male production and a shift in male broods from earlier broods to later broods near the end of the adult life cycle. For example, sex ratios in control daphnids ranged from 0.43 to 0.67 in broods 3–4 compared with 0.0–0.13 in daphnids exposed to the residential oxidation lagoon. Secondary sexual characteristics of both sexes were statistically significantly increased by the sewage lagoon effluent but not the municipal effluent. These preliminary results suggest that alteration in timing of sexual determination due to exposure to sewage treatment plant effluents could severely impact the survival of daphnid populations.



## 1. Introduction

Daphnids are important invertebrate organisms that serve as dominant herbivores and a food source for larval fish in aquatic ecosystems. Most daphnids are cyclic parthenogenetic species that exhibit both asexual and sexual reproduction (Dodson and Frey, 1991). Certain environmental cues such as reduced photoperiod, low food levels, and high density have been shown in laboratory settings to trigger male offspring production (Hobæk and Larsson, 1990; Kleiven et al., 1992). The survival strategy is to shift to sexual reproduction and produce sufficient numbers of resting or winter eggs called ephippia. These eggs are haploid and presumably require the presence of males for fertilization. These eggs then enter a diapause stage and are capable of surviving adverse environmental conditions during winter. Recent studies have demonstrated that male determination is modulated by the juvenile hormone methyl farnesoate (Olmstead and LeBlanc, 2002, 2003). Methyl farnesoate apparently transduces the environmental cues to the physiological response of male sex determination during a specific 12-h period of ovarian egg development (Olmstead and LeBlanc, 2002).

Several studies have reported modulation in male offspring production following exposure to environmental chemicals (Olmstead and LeBlanc, 2003; Oda et al., 2005; Wang et al., 2005). Most chemicals that stimulate male production have strong juvenoid agonist activity (i.e., juvenile hormone III, pyriproxyfen, and fenoxycarb) (Wang et a., 2005). Other chemicals (trans-retinoic acid, kinoprene, 4-nonylphenol, and bisphenol A) were shown to potentiate the activity of methyl farnesoate. Only a few chemicals have been shown to reduce male offspring production or alter the timing of male production (Baer and Owens, 1999; Kashian and Dodson, 2004). The elimination of sexual reproduction may enhance the accumulation of deleterious mutations in the population genome (Paland and Lynch, 2006).

Perturbations in sexual development in daphnids have also been used as an endpoint following exposure to endocrine disrupting chemicals. The development of sexual characteristics in daphnids apparently involves hormonal processes during juvenile development and maturation (Mitchell, 2001). Olmstead and LeBlanc (2000) demonstrated that both DES and methoprene increased the length of the abdominal process (a secondary sexual characteristic in females) relative to body size. Androstenedione, a steroidal vertebrate androgen, stimulated the normalized growth of the first antennae, a secondary sexual characteristic of male daphnids. These and other studies demonstrate that secondary sexual characteristics can be modulated by environmental contaminants (Gerritsen et al., 1997; Gibble and Baer, 2003).

Recent reports have brought attention to the presence of pharmaceuticals and personal care products in untreated and

☆ *Funding sources*: The University of Louisiana at Monroe, College of Pharmacy.
*Animal welfare statement*: Studies involving experimental animals were conducted in accordance with national and institutional guidelines for the *protection of human subjects and animal welfare*.

* Corresponding author. Fax: +1 318 342 3037.
*E-mail address:* pybaer@ulm.edu (K.N. Baer).

0147-6513/$ - see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.ecoenv.2008.12.007

sewage treatment plant effluents (STPs) as well as surface waters (Daughton and Ternes, 1999; Smital et al., 2004; Schwab et al., 2005). These effluents contain complex mixtures of estrogens or estrogen-mimicking compounds including estradiol, estrone, 17α-ethinylestradiol, phthalates, pesticides, and pharmaceuticals. The majority of the research has focused on the impact of reproductive endocrine disruptors from STPs on teleost species (Jobling and Sumpter, 1993; Purdom et al., 1994; McMaster et al., 1991; Edwards and Guillette, 2007). However, there is growing evidence of the effects of environmental estrogens on invertebrate species, although the toxicity exerted by these chemicals likely results from non-endocrine-mediated effects (Clubbs and Brooks, 2007). Several invertebrate studies (summarized in Clubbs and Brooks, 2007) reported reproductive and developmental abnormalities with 17α-ethinylestradiol including smaller gnathopods and histological aberrations of the reproductive tract in the amphipod *Hyalella azteca* (Vandenbergh et al., 2003), and earlier emergence times in first and second generations of the midge *Chironomus riparius* (Watts et al., 2003).

Little information is currently available on the potential impacts of mixtures of bioactive compounds in STPs to invertebrate species such as daphnids. It is plausible that any chemical capable of impacting an organism's fitness, such as sex determination, sexual maturation, or fecundity may have significant consequences for the ecosystem (Kashian and Dodson, 2004). Based on these considerations, the purpose of the current study was to investigate the modulation of sex ratios, sexual maturation, molting, and growth in *Daphnia magna* following multigenerational exposure to STPs.

## 2. Materials and methods

### 2.1. Effluent collection and chemicals

Grab samples were collected in 20 L Nalgene® polypropylene carboys twice weekly from the outfall of a municipal sewage treatment plant effluent (MSTP) serving Monroe, LA (population approximately 52,000) and a residential oxidation lagoon (ROL; serving approximately 1000). The MSTP uses primary, secondary, and tertiary treatment consisting of activated sludge treatment followed by ultraviolet light and chlorination. The ROL uses one anaerobic and one aerobic lagoon followed by a rock-plant filter and ultraviolet light treatment. Samples were transported to the laboratory and sequentially filtered using nylon mesh (210, 60, and 20 μm, respectively). Samples were either used immediately or stored at 4 °C (⩽96 h). Methyl farnesoate (⩾95%) was purchased from Echelon Biosciences, Inc. (Salt Lake City, UT).

### 2.2. Daphnid cultures

*D. magna* Straus cultures (clone 5 obtained from The Academy of Natural Sciences, Philadelphia) were held in 150-mL glass beakers (1 adult daphnid/beaker) containing ~100 mL of H–H COMBO medium (Baer and Goulden, 1998). The temperature was maintained at 20±2 °C and a 16-h light:8-h dark photoperiod (illumination ranged between 100 and 115 lux) was employed. The medium was renewed and daphnids were fed a green algae species, *Ankistrodesmus falcatus* (250,000 cells/mL), three times weekly (i.e., MWF). Representative water quality characteristics were as follows: pH, 7.42±0.04; dissolved oxygen (mg/L), 7.45±0.66; conductivity (μmho s), 400±70; total hardness (mg/L as $CaCO_3$), 108±6. These conditions allowed for continuous parthenogenetic reproduction in laboratory cultures.

Separate daphnids were cultured in either MSTP or ROL effluents for greater than 10 generations under conditions that allow for parthenogenetic reproduction described above. Each subsequent generation was started with neonates from the third or fourth brood.

### 2.3. Influence of sewage treatment effluents and food levels on male offspring production

To evaluate the influence of different exposure treatments on the sex ratio of *D. magna* offspring, randomly selected neonates from either laboratory cultures or treated sewage effluent cultures (age <24 h old, ⩾third brood, 1 daphnid/beaker) were used. All experiments were conducted in an environmental chamber with constant temperature (20±2 °C) and 8-h light/16-h dark photoperiod (48–55 lux). The solutions were renewed and daphnids fed *A. falcatus*, at concentrations of 50,000 cells/mL three times weekly (i.e., MWF). The combination of reduced photoperiod and low feeding rate has been shown in the laboratory to stimulate male production in *D. magna* (Baer and Owens, 1999). Daphnids were individually held in 150-mL glass beakers containing 100 mL of sewage treatment effluent. All studies were allowed to continue until near the end of the adult's life cycle (6–8 broods; 35–45 days). During the experiments, temperature, dissolved oxygen, pH, conductivity, alkalinity, hardness, ammonia, nitrate, chloride, phosphate, and turbidity were monitored weekly. The sex and morphology of neonates were observed and counted using a dissecting microscope. Male daphnids were identified by the presence of large, prominent first antennae. The sex ratio was determined as the total number of males divided by the total number of neonates in a brood. Additional endpoints included the total number of molts, total number of live neonates (fecundity), and adult length at test conclusion. Total body length was measured from the apex of the head to the base of the shell spine under 40× magnification using an ocular micrometer and imaging analysis software.

A separate study was conducted to determine if an increase in energy reserves could reduce male offspring production similar to sewage treatment effluent exposures. The influence of food levels and reduced photoperiod on daphnid sex ratios was investigated using 50,000 and 250,000 cells/mL of *A. falcatus* (2 and 6 mg/L dry mass, respectively). Experimental conditions were as previously described.

Experiments were conducted to evaluate the potential for STPs to stimulate the growth of indigenous algae and/or bacteria and provide an additional food source to daphnids. Filtered samples were inoculated with *A. falcatus* (50,000 cells/mL) and incubated for 72 h under experimental conditions (8-h dark/16-h light, 20 °C). In addition, 1 mL aliquots of treated sewage effluents were added to COMBO algae medium and placed under continuous light (~2000 lux) for 72 h. At the end of the period, algal cell densities were determined using a hemacytometer. ROL and control samples were also analyzed for total aerobic bacteria and total coliform bacteria using a paddle test (Hach Chemical Co.).

### 2.4. Secondary sexual characteristics

Male and female sexual maturation was evaluated following exposure to STPs. Males are distinguished from females by differences in secondary abdominal processes, body length, rostrum, first antennae, breast margin, and first leg. Male and female daphnids from multigenerational exposures to control, MSTP, and ROL under conditions that stimulate male offspring production were separated and allowed to grow to the adult stage (~14 days). The length of the secondary abdominal process in females and the first antennae in males was measured under 40× magnification using an ocular micrometer and imaging analysis.

### 2.5. Stimulation of male offspring production by methyl farnesoate in the presence of residential oxidation lagoon effluent

Wang et al. (2005) developed an *in vivo* screening assay for detecting juvenoid or anti-juvenoid activity of chemicals using *D. magna*. A modification of this assay was used to determine the modulatory effect of ROL on male offspring production stimulated by MF exposure under conditions that support parthenogenetic reproduction. MF was prepared in absolute ethanol and used immediately or stored at 4 °C between renewals. Ethanol controls (0.02%) did not produce males. Adult female daphnids (7–10 days, first presence of eggs in brood pouch) from laboratory cultures and ROL cultures were randomly assigned to controls and 50 and 200 μg/L MF for 48 h. Daphnids were housed in 150 mL glass beakers containing 100 mL of solution (COMBO medium or ROL) and fed *A. falcatus* (150,000 cells/mL). At the end of the 48-h exposure period, daphnids were transferred to their respective solutions without MF and the sex ratios of the next three broods were determined as previously described. The temperature was maintained at 20±2 °C and a 16-h light:8-h dark photoperiod (illumination ranged between 100 and 115 lux) was employed.

### 2.6. Statistical analysis

Statistical comparisons between exposure groups were accomplished using a paired *t* test. Additional non-parametric tests (Wilcoxon rank sums test) were employed when the data were heterogeneous (Bartlett's test) and/or not normally distributed (Shapiro–Wilk's test). All tests were performed using standard software (JMP IN, SAS Institute, Inc., Cary, NC, USA).

## 3. Results

### 3.1. Influence of treated sewage effluents and food levels on male offspring production

Water quality characteristics of COMBO, MSTP, and ROL are presented in Table 1. Both STPs had lower hardness levels than