ABSTRACT

Multigenerational Responses of *Daphnia magna* to Ethynylestradiol and Faslodex

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Select environmental contaminants can disrupt normal functions of aquatic invertebrate endocrine systems. While ecological risk assessments often rely on standardized laboratory toxicity tests to assess ecological impacts, these techniques may not be appropriate for endocrine active compounds, including select pharmaceuticals in the environment. Subsequently, multigenerational designs are recommended to assess organismal responses to low-level exposures to these compounds. The objective of this study was to investigate effects of a mammalian estrogen receptor agonist and antagonist on endocrine biomarkers and transgenerational life-history parameters of a model invertebrate, *Daphnia magna.*

Results from this study suggest that the pharmaceuticals, Ethynylestradiol and Faslodex, model therapeutics designed to interact with vertebrate estrogen receptors, did not act through the ecdysone receptor in *D. magna*. Thus, toxicity Ethynylestradiol and Faslodex exerted on *D. magna* likely resulted from non-endocrine-mediated responses, which may reduce uncertainty in future assessments of invertebrate responses to endocrine active pharmaceuticals in the environment.

Multigenerational Responses of *Daphnia magna* to Ethynylestradiol and Faslodex

by

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A Thesis

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ABBREVIATIONS

20HE	20-hydroxyecdysone
AchE	Acetylcholinesterase
ACRs	Acute:Chronic Ratios
ANOVA	Analysis of Variance
BSA	Bovine Serum Albumin
CYP	Cytochrome P-450s
DO	Dissolved Oxygen
EE2	Ethynylestradiol
EIA	Enzyme Immunoassay
ELISA	Enzyme-Linked Immunosorbent Assay
F	Faslodex
F0	Parental Generation
F1	Neonates of the Parental Generation
LC50	Lethal Concentration to 50% of Test Organisms
LOEC	Lowest Observable Effect Concentration
NC	Not Collected
NOEC	No Observable Effect Concentration
PBS	Phosphate Buffered Saline
PBST	PBS + 0.05% Tween 20
PVDF	Polyvinylidene difluoride
r	Intrinsic Rate of Population Growth

RHW	Reconstituted Hard Water
SERMs	Selective Estrogen Receptor Modulators
SD	Standard Deviation
SDS	Sodium dodecyl sulfate
T	Testosterone
Vt	Vitellin
WWTP	Wastewater Treatment Plant

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CHAPTER ONE

Introduction

In recent years there has been considerable concern and growing evidence over the potential for anthropogenic chemicals in the environment to interfere with endocrine systems. The endocrine and nervous systems comprise an internal communication pathway that regulates all responses and functions of the body (Rang et al. 1995). The endocrine systems in humans and wildlife contain a network of glands that produce and secrete hormones, chemical messengers that travel through the bloodstream to specific receptors where they act to initiate essential biological responses in target tissues (Rang et al. 1995). Growing evidence suggests that synthetic, and some naturally occurring, chemicals in the environment are disrupting or modulating the normal functions of endocrine systems. This "disruption" occurs as the endocrine-modulating compound interacts with steroid hormones and their receptors or other hormones and transcription factors in the biochemical pathway of hormonal activity (Kavlock 1999).

Intense efforts are currently underway to identify endocrine-modulating chemicals and assess their effects on ecological receptors. However, endocrine-active pharmaceuticals represent a group of emerging, environmental contaminants that are often distributed before ecotoxicological information is available or fully investigated. Because of the potential for exposure to low levels of these compounds to modulate endocrine function of aquatic organisms, potential current and future environmental hazards are evaluated using various ecotoxicology techniques and experiments. While mammalian pharmacological safety information may allow for risk predictions of

endocrine active pharmaceuticals to fish and other vertebrates (Huggett et al. 2003), a lack of understanding of invertebrate endocrinology (LeBlanc et al. 1999) precludes extending similar predictions to invertebrates at this time (Stanley et al. 2006, In Press). If the potential invertebrate impacts of mammalian estrogen-receptor-active therapeutics are to be assessed, then an understanding of invertebrate endocrinology and responses to these compounds under realistic environmental exposure scenarios is required. Here, appropriate selection of and experimentation with model compounds with characterized mechanisms of action in vertebrates and model organisms of ecological importance may provide valuable information to reduce uncertainty associated with ecological risk assessments of endocrine active pharmaceuticals in the environment.

A similar approach was taken in a study to identify possible physiological and biochemical target sites for the estrogenic effects on *D. magna* resulting from exposure to the model environmental estrogen diethylstilbestrol (DES) (Baldwin et al. 1995). DES is a synthetic estrogen that was prescribed to prevent miscarriage between 1938 and 1971 in the United States. In 1971, the U.S. Food and Drug Administration issued a warning about the use of diethylstilbestrol during pregnancy after a relationship between exposure to this synthetic estrogen and the development of clear cell adenocarcinoma of the vagina and cervix was found in young women whose mothers had taken diethylstilbestrol while they were pregnant. While the effects of such estrogens on vertebrates are known, few studies have investigated their role in invertebrates. Baldwin et al. (1995) found that chronic exposure of daphnids to 0.50 mg L^{-1} DES reduced molting frequency in first generation juveniles, decreased fecundity in second generation juveniles, and altered steroid metabolic capabilities.

Ethynylestradiol (EE2), another model synthetic estrogen, is the most potent estrogenic component of oral contraceptives (Kozak et al. 2001), which are among the most widely prescribed pharmaceuticals (RxList 2002). There has been a broad range of concentrations of EE2 reported in raw sewage (500-2250 ng L^{-1}), effluent of wastewater treatment plants (WWTP; <0.2-1780 ng L^{-1}), and in surface waters of several countries (0.1-15 ng L^{-1}) (Kozak 2001). It appears that EE2 and other pharmaceuticals are potentially released in a continuous fashion from WWTPs. Because EE2 was designed to be potent at the vertebrate estrogen receptor, exposure to low concentrations has the potential to disrupt endocrine and reproductive functions in wildlife (Foran et al. 2002). Although effects of EE2 have been widely examined in vertebrates and select invertebrates (Table 1), limited studies have assessed potential multigenerational effects of EE2, which are more representative of environmentally realistic exposure scenarios. Even less information is available for multigenerational EE2 exposure to invertebrates.

EE2 has the potential to disrupt normal physiological functions in vertebrate organisms (Kozak 2001), as documented by sex reversal, decreased female fecundity, aromatase expression in gonads, and the development of gonadal alteration or testis-ova in adult male Japanese medaka (Metcalfe et al 2001; Scholz et al. 2000). In adult male trout, as little as 2 ng L^{-1} EE2 induced vitellogenin and inhibits testicular growth (Jobling et al 1996) and exposure to 0.1 ng L^{-1} of EE2 for 90 days caused developmental alterations of gonadal morphology in the testis of medaka (Metcalfe et al 2001). In mammals, EE2 treatment resulted in a decrease in sperm motility associated with declining testosterone (Kaneto et al 1999).

Table 1. Review of literature on ecotoxicity of EE2 in invertebrates.

Study Information				Endpoints					Response
Author(s)	Date	Duration	Organism	Survival	Reproduction	Sexual differentiation	Growth / Development	Other	
Hutchinson et al.	1999	21 days	*Tisbe battagliai*	✔	✔		✔		no significant impact at highest concentration, 100 μg L^{-1}
Anderson et al.	2001	48 hr, 5 days	*Acartia tonsa*	✔			✔		LC50 = 1.1 mg L^{-1} EC50=0.088 mg L^{-1}
Brietholz, Bengtsson	2001	18 days	*Nitocra spinipes*	✔	✔	✔	✔		no significant impact at highest concentration, 0.05 mg L^{-1}
Watts, Pascoe, Carroll	2001	2 generations	*Chironomus riparius*	✔	✔	✔		emergence times	at 1 ng L^{-1}, 1st and 2nd generation emerged significantly earlier; Percent emergence decreased in 2nd generation
Meregalli, Ollevier	2001	9 days	*Chironomus riparius*	✔			✔		no adverse effect on larvae tested
Radix et al.	2002	3 days	*Brachionus calyciflorus*		✔	✔			intrinsic rate of population growth increased as the number of females in population decreased at concentrations higher than 1.72 mM L^{-1}
Watts, Pascoe, Carroll	2002	100 days	*Gammarus pulex*	✔		✔	✔		significantly more females at concentrations higher than 1 mg L^{-1}

Table 1 - *Continued*

Study Information				Endpoints					Response
Author(s)	Date	Duration	Organism	Survival	Reproduction	Sexual differentiation	Growth / Development	Other	
Vandenbergh et al.	2003	2 generations	*Hyalella azteca*			✔	✔		2nd generation males developed significantly smaller gnathopods; histological aberrations of the reproductive tract in F2 males
Watts, Pascoe, Carroll	2003	through 4th instar	*Chironomus riparius*				✔		Delayed moulting and reduced larval weights at 1 mg L^{-1}; mouthpart deformaties observed at 10 ng L^{-1}
Segner et al.	2003	partial life-cycle and multigeneration	*Hydra vulgaris, Gammarus pulex, Chironomus riparius, Hyalella azteca, Lymnaea stagnalis*		✔		✔		Most effects induced at higher concentrations; low dose effects observed in full life cycle tests, particularly in the 2nd generation
Goto, Hiromi	2003	48 hr, 25 days	*Daphnia magna*	✔	✔	✔	✔	ability to swim	Norethindrone inhibited swimming at >3 ppm, EE2> 100 ppb decreased number of offspring to 75%, mixture (EE2 5.88 ppb, NOR 94.12 ppb) also decreased offspring number by 57%
Roepke et al.	2005	96-hr	*Stronglyocentorus purpuratus, Lytechinus anamesus*				✔		Inhibited normal development of embryos at low ng $L^{-1}$with significant inhibition (15-20% abnormal) around 0.1 ng mL^{-1}

EE2 has also been experimentally shown to adversely affect invertebrates. In a multigenerational study with the amphipod *Hyalella azteca*, second-generation male exposed from gametogenesis until adulthood to 0.1 and 0.32 μg L^{-1} developed significantly smaller second gnathopods (Vandenbergh et al. 2003). Histological aberrations of the reproductive tract of post-F1-generation males were also observed in all exposure concentrations, 0.1 to 10 μg L^{-1} (Vandenbergh et al. 2003). Chronic exposure of EE2 in *Chironomus riparius* resulted in significantly earlier emergence times in first and second generations at 1 ng L^{-1} of EE2 (Watts et al. 2001) and at 10 ng L^{-1} mouthpart deformities were observed (Watts et al. 2003).

ICI 182,780, or Faslodex (F), is a novel, model steroidal antiestrogen designed for use in human breast cancer treatment. Endocrine therapy is important in the management of all stages of breast cancer, and antiestrogen therapy remains a highly used and effective treatment for endocrine-responsive breast cancers (Clarke et al. 2001). Other nonsteroidal antiestrogens, or selective estrogen receptor modulators (SERMs), such as Tamoxifen and its derivatives, are well established as effective breast cancer treatments for their antagonistic effects on the estrogen receptor (Howell et al. 2000). However, the partial agonist activity of Tamoxifen on the uterus and the development of drug resistance have limited its clinical utility (Howell 2001). F is one of the first identified agents in a new class of antiestrogens, estrogen receptor down-regulators, and is devoid of agonist activity according to preclinical models and clinical trials (Howell et al. 2000). This “pure” antiestrogen completely suppresses the effects of estrogens as the result of disrupted nuclear localization of ligand-estrogen receptor complexes and increased proteolytic degradation of the estrogen receptor (Howell et al. 2000). Due to its potency

and specific target response, F is showing promising clinical activity in the treatment of advanced breast cancer after Tamoxifen therapy (Howell 2001).

While F is currently still undergoing testing, its clinical potential indicates it could be prescribed to a level as great as its predecessor Tamoxifen, which is currently listed as one of the most widely prescribed pharmaceuticals in the U.S. (RxList 2002). Beyond clinical pharmokinetics, much is currently unknown about how the drug interacts with and changes within the target organism, the disposition of F in wastewater treatment plants and the environment, and the potential to adversely affect aquatic organisms. Only limited information is currently available in literature on the specific effects of antiestrogens on reproduction in fish and invertebrates. For example, the few experimental studies of antiestrogens in fish involve almost exclusively analogs of Tamoxifen or its metabolites (Byung-Ho et al.2003; Lazier et al. 1996; Leanos-Castaneda et al. 2002; Hornung et al. 2003). One study exposed juvenile fathead minnows to pure antiestrogen ZM 189,154 for 21 days at the mean measured concentrations of 5.0, 24.4, and 76.6 $\mu g\ L^{-1}$ (Panter et al. 2002). A significant decrease in vitellogenin formation was observed at all exposure levels after 14 days of exposure, while there were no significant changes in body weight or length of the fish (Panter et al. 2002). The few experimental studies of F in invertebrates were published during the experimental and data analyses phases of this Thesis. One such study noted the inhibited development of sea urchin embryos at concentrations as low as 0.03 $ng\ L^{-1}$ (Roepke et al. 2005). Another study exposed *D. magna* adults for 6 days at F concentrations ranging from 1 to 100 $\mu g\ L^{-1}$. There were no significant effects found on the observed endpoints, including survivorship, fecundity, ephippium production, adult size, changes in morphology, and

sex determination of neonates produced during the short-term exposure (Kashian and Dodson 2004).

Both EE2 and F have been extensively investigated in required clinical trials for their effects in the desired target organism and related organisms, but the toxicological information in nontarget organisms, especially invertebrates, is not as prevalent for both compounds. Chemicals that exhibit estrogenic or androgenic activity in vertebrates may also affect invertebrate endocrine systems controlling reproduction and development (Kahl et al. 1997). Since over 95% of animal species are invertebrates, it is important to consider non-vertebrate hormonal systems as potential targets for endocrine disruption (LeBlanc et al. 1999). Despite a larger availability of vertebrate endocrine disruption data in literature, significant physiological differences limit the potential for extrapolation of endocrine disruption data from vertebrates to invertebrates (Hutchinson 2002).

Several studies have identified certain parameters in invertebrates that are affected by environmental estrogens; however, it has not been possible to conclude categorically that the effects are hormone-mediated and result from an interaction with the endogenous endocrine system of invertebrates (Segner et al. 2003; Hutchinson 2002). A basic conceptual model of the neuroendocrine system, examples of major hormones, and the processes they control are presented in Figure 1. Ecdysteroids are invertebrate steroid hormones involved in the initiation of molting and egg maturation in many invertebrates (Figure 1) (LeBlanc et al. 1999). If compounds designed to act on the vertebrate estrogen receptor (EE2, F) were acting through the invertebrate ecdysone receptor, it is expected that the processes specifically under the control of ecdysteriods, reproduction and molting, would be significantly affected.

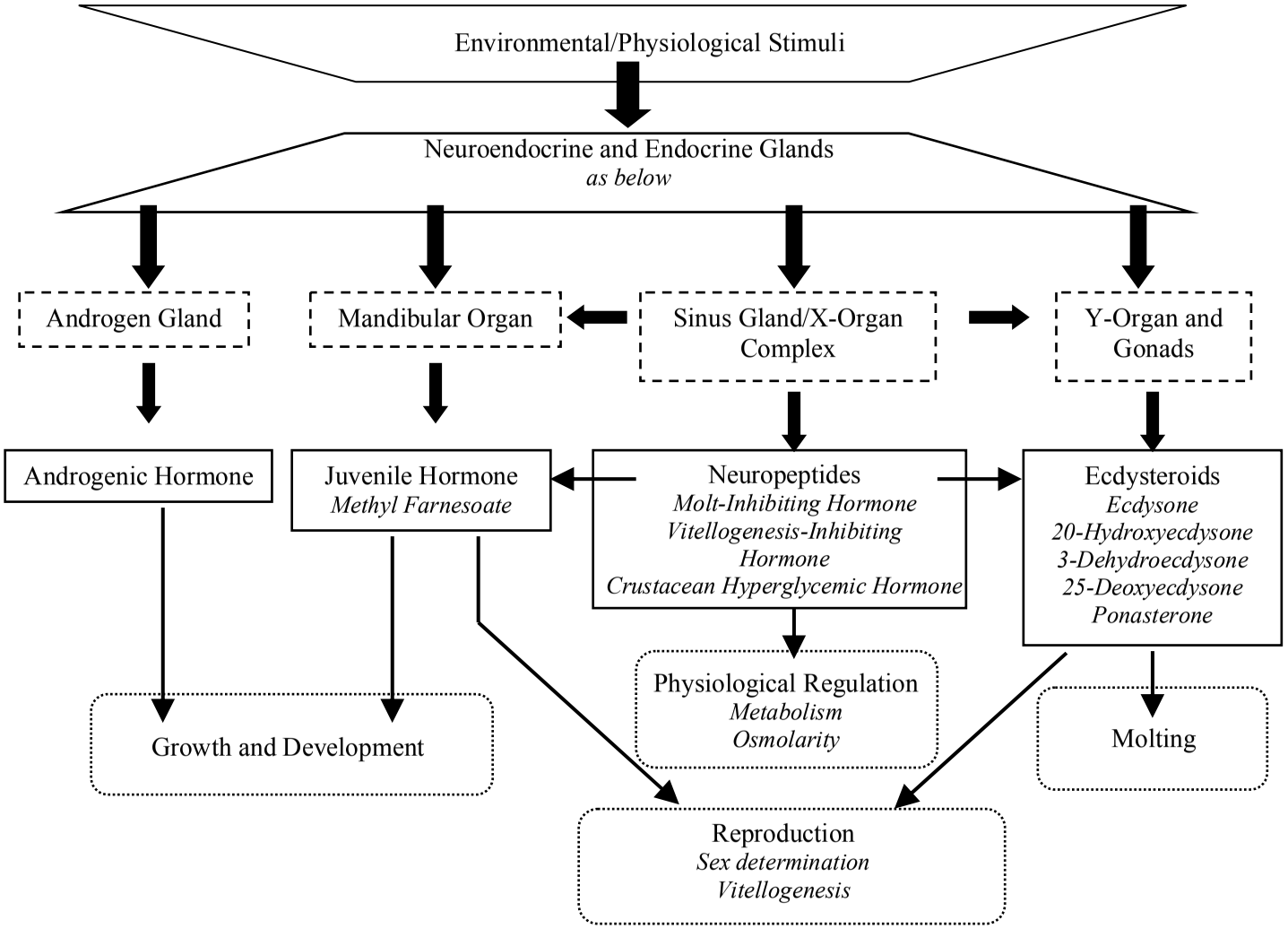

Fig. 1. Conceptual model of the neuroendocrine system, examples of major hormones, and the processes they control in Crustacea.

There is a diverse literature on the developmental and reproductive effects of mammalian endocrine disrupting chemicals in Crustacea, although there is growing evidence that such effects may not be mediated via Arthropod hormone systems (Hutchinson 2002). Attempts to identify the presence and role for estrogens in invertebrate development or reproduction have met with positive results, although inconsistencies exist between Phyla and a functional role in Crustaceans, such as *D. magna*, has not yet been established (Baldwin et al. 1995). Some results identify physiological and biochemical parameters in invertebrates, including *D. magna*, that may be perturbed by environmental estrogens or other endocrine disruptors (Oetken et al. 2004). However, it remains to be established whether the estrogen-induced alterations in the invertebrate species do indeed result from disturbance of the endocrine system (Segner et al. 2003).

Crustaceans and other arthropods utilize ecdysteroids as major endocrine signaling molecules (Figure 1) (LeBlanc et al. 1999), and interference with ecdysteroid production or function could provide a means by which environmental contaminants such as F and EE2 impact crustaceans (Mu and LeBlanc 2002a). For example, exposure of *D. magna* to 8.0 μM (2.31 mg L^{-1}) T had no significant effect on parental survival, but did delay molting, significantly reduced the number of viable offspring produced, and caused a significant incidence of developmental abnormalities among offspring (Mu and LeBlanc 2002b). It is documented that developmental abnormalities induced by antiecdysteroids, including T, can be associated with suppressed ecdysone levels in embryos and these abnormalities can by prevented by co-exposure to 20HE (Mu and LeBlanc 2002a; Mu and LeBlanc 2002b). Exposure of *D. magna* embryos to 20HE alone

had no discernable effect on embryo development (Mu and LeBlanc 2002a). Another recent study acutely exposed *D. magna* adults and chronically exposed *D. magna* from neonate to adult for 25 days at T concentrations ranging from 1-100 μg L^{-1} (Kashian and Dodson 2004). Specifically, Kashian and Dodson (2004) reported that short-term T exposure at 100 μg L^{-1} significantly reduced fecundity while long-term exposure did not produce the same effect, potentially indicating T hydroxylation with long-term exposure. Ecdysteroids have a documented, significant role in the regulation of critical processes in daphnid embryo development and environmental antiecdysteroids, such as T, can disrupt normal development.

While ecological risk assessments rely on standardized laboratory responses (e.g., survival, growth, reproduction) to assess ecological effects, these techniques may not be appropriate for endocrine active compounds. Because of the potential for continuous exposure of these compounds at low levels in nontarget aquatic organisms, it is more relevant to perform chronic, life-cycle type tests that allow for assessment of long-term reproductive effects and encompass sensitive stages of organism development, subsequently decreasing hazard uncertainties in ecological risk characterization (Segner et al. 2003). Multigenerational tests that observe long-term reproductive effects are also recommended for endocrine disrupting compounds because they provide a means of evaluating ecologically relevant population effects (Taylor et al. 1999; Sanchez and Tarazona 2002; Patyna et al. 1999; Hutchinson 2002). However, transgenerational studies with invertebrates and such compounds are limited. Also, the possibility to detect an effect due to endocrine modulation is greatest in tests using the full life-cycle where various processes (development, growth, molting, reproduction) are controlled by the

endocrine system and therefore potential targets for disruption (Segner et al. 2003). This possibility is recognized in the recommendation that full life cycle tests be adopted as the "gold standard" for assessment of endocrine disrupting compounds in invertebrates (Ingersoll et al. 1999). Although effects of EE2 and F have been examined in vertebrates and select invertebrates, limited studies have assessed potential multigenerational effects.

Objectives

The objective of this study was to investigate the chronic effects of a mammalian estrogen receptor agonist (EE2) and antagonist (F) on the biochemistry and transgenerational life-history of a model invertebrate, *Daphnia magna.* This investigation was not designed to identify the mechanisms of action of EE2 or F in invertebrates, but to examine the multigenerational responses of *D. magna* to compounds that are potent at mammalian estrogen receptors. Several factors make *D. magna* an attractive model species for multigenerational toxicity studies. *D. magna* was chosen for study because they are ecologically significant in many food webs, readily cultured in the laboratory, a common research organism, and are included in regulatory assessments of pharmaceuticals (Taylor et al. 1999; Depledge and Billinghurst 1999; Hutchinson 2002; LeBlanc et al. 1999).

Given the uncertain nature of daphnid endocrine systems, normal hormonal functioning, and the mechanisms by which these and other pharmaceuticals exert their toxicological action in invertebrates, research of the multigenerational effects of compounds designed to act on vertebrate estrogen receptors is warranted. The structural similarity of vertebrate estrogens and ecdysone points to the possibility that estrogenic compounds could interfere with endogenous steroids in invertebrates (Segner et al. 2003).

Some studies indicate the possibility of interaction between estrogenic chemicals and the ecdysteroid receptor (Zou and Fingerman 1997; Baldwin et al. 1995; Segner et al. 2003). Others have demonstrated that testosterone, which is structurally similar to estrogen, elicits embryo toxicity to daphnids by interfering with ecdysteroid activity (Mu and LeBlanc 2002b; Mu and LeBlanc 2004). It is my hypothesis that EE2 and F exposure over two generations will affect *D. magna* in a dose dependent fashion by reducing somatic growth, fecundity, number and sex ratios of viable neonates, population growth rate, and modulating vitellin and ecdysone levels as might be expected in compounds acting through the ecdysone receptor.

CHAPTER TWO

Materials and Methods

20-Hydroxyecdysone and Testosterone

In examining the effects of an estrogen receptor agonist (EE2) and antagonist (F) on *D. magna*, additional chemicals with known impacts on normal physiological and reproductive functioning were chosen to serve as positive controls. 20-hydroxyecdysone (20HE) and Testosterone (T) were chosen because of their specific mechanisms of action (not working through the estrogen receptor, where EE2 and F potentially act, but through the Ecdysone Receptor) and documented chronic effects on *D. magna*. 20HE is a steroid that serves primarily as an endogenous molting hormone in Crustacea, including *D. magna*, but also serves in the control of reproduction and embryogenesis (Subramoniam 2000). T is a vertebrate steroid hormone shown to have anti-ecdysteroidal activity in *D. magna* (Mu and LeBlanc 2002b; Mu and LeBlanc 2004).

Because 20HE and T act through the invertebrate Ecdysone Receptor in *D. magna*, these compounds had a critical role in this present study. Observing the multigenerational effects of 20HE and T allowed for the comparison of the effects of EE2 and F, in which the mechanism of action is unknown, to compounds with known mechanisms of action. For example, it was my hypothesis that high concentrations of EE2 would have significant effects on the reproduction and development of *D. magna*. While the multigenerational effects of EE2 alone require understanding, comparing these results to the structurally-similar and ecdysone receptor antagonist T could potentially contribute to an understanding of the mechanism of action of EE2 in *D. magna*. As

20HE and T have documented chronic effects in *D. magna*, these compounds also served as positive controls for examining responses to EE2 and F.

Experimental Compounds

EE2 and 20HE were purchased from Sigma Chemical (St. Louis, MO, USA), F was purchased from Tocris (Ballwin, MO, USA), and T was purchased from Alltech Associates (Deerfield, IL, USA). All solutions were prepared using reconstituted hard water (RHW) (American Public Health Association 1995). Because these compounds have limited solubility in water, stock solutions were prepared with appropriate solvent carriers. Two controls were utilized in each test, one with the appropriate solvent carrier and one consisting only of RHW. EE2 (Sigma: E4876), F (Tocris 1047), and 20HE (Sigma H5142) were dissolved in acetone and diluted with RHW to give a final solvent concentration of <0.01% T (Alltech 4873) was purchased as 1 mg mL^{-1} methanol, the methanol evaporated using a Zymark TurboVap® LV nitrogen evaporator (Hopkinton, MA, USA) at 30^{o}C, 15 psi, the testosterone dissolved in a smaller volume of methanol, and finally diluted with RHW to give a solvent concentration of <0.01%. All stock solutions were stirred for 24 hours at 4^{o}C, in the dark, before diluted to give final treatment levels.

Experimental Organisms

Less than 24 hours old *D. magna* were selected for all experiments from a stock culture maintained for approximately 1 year at Baylor University's Ecotoxicology and Aquatic Research Laboratory (Waco, TX, USA). Daphnids were mass-cultured as previously described (Turner et al. 2001; Hemming et al. 2002). Organisms were fed a

1.2 mL algae-Cerophyll® suspension once daily (Knight and Waller 1992; Hemming et al. 2002). Water was renewed every other day by static renewal to maintain water quality (USEPA 1996; OECD 1998). Adults were observed daily and neonates counted every other day to provide a record of fecundity and monitor of the overall health of the culture.

Experimental Design

Daphnids in each multigeneration study were exposed to five sublethal treatment levels, a reconstituted hard water control, and a carrier control. Sublethal concentrations were chosen following preliminary range-finding toxicity tests. Acute toxicities were assessed in static-renewal tests generally according to the standard protocols for *D. magna* 96 hr acute tests (USEPA 1996; OECD 1998). Multigeneration studies with EE2, F, T, and 20HE followed an identical experimental design (Figure 2).

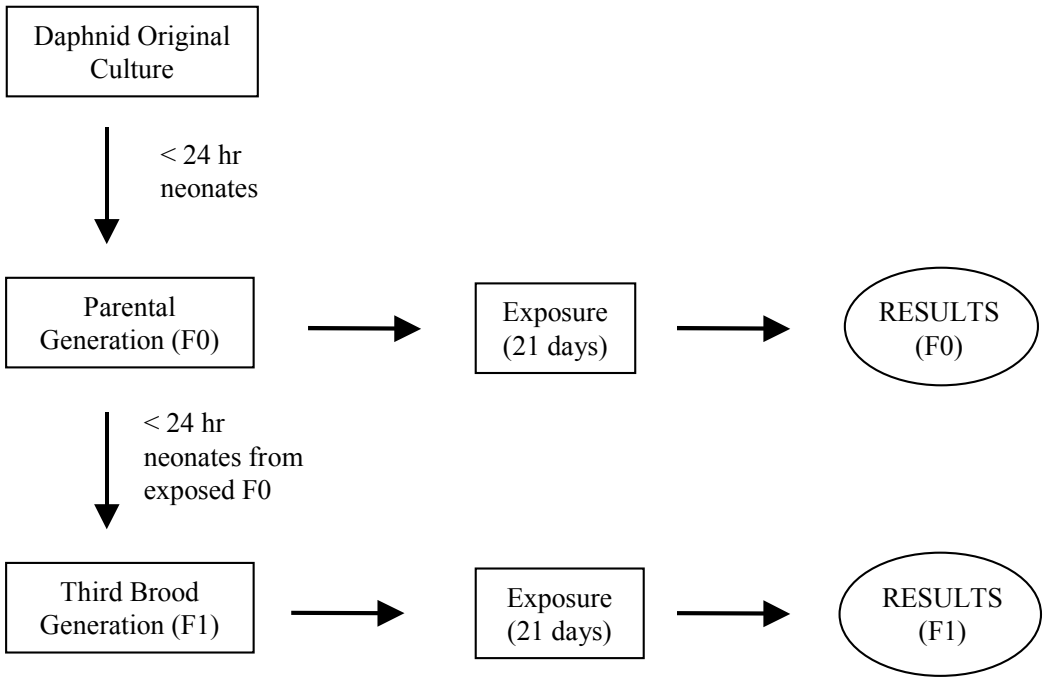

Fig. 2. Conceptual model of experimental activities in multigeneration toxicity studies with *D. magna.*

Chronic toxicities were assessed in static-renewal tests generally according to the standard protocols for *D. magna* 21-day reproduction tests (USEPA 1996; OECD 1998). In the parental generation (F0) reproduction tests, 10 neonates (<24 hours old) per concentration were individually transferred from the original culture to 10, 100-mL tall form glass beakers containing 50 mL of the test medium.

For the second generation portion of each sudy, 10 neonates (< 24 hours old) from the third brood of the parental generation (F0) were randomly collected from each exposure concentration and individually transferred to 10, 100 mL tall form glass beakers containing 50 mL of the test medium. These neonates (F1) were exposed to the same treatment levels of the test chemical as F0 mothers.

All multigeneration studies were performed in reconstituted hard water (RHW) (American Public Health Association et al. 1995). All studies were renewed every other day (OECD 1998). Because aqueous stabilities of the test substances were generally unknown, new stock solutions were prepared weekly and stored in the dark at 4^{o}C between static renewals. Daily activities of each multigeneration test are summarized in Appendix A.

Water Chemistry

For each study, dissolved oxygen, temperature, and pH were measured once a week in the control and in the highest test concentration. Measurements of dissolved oxygen and temperature were taken using a YSI Model 55 handheld DO and temperature system; pH was measured using a VWR SR601C pH meter. Alkalinity and hardness of freshly prepared reconstituted hard water used in the test medium were determined by

amperiometric and colorimetric titration, respectively (American Public Health Association et al. 1995).

Response Variables

This study examined standard *D. magna* endpoints (e.g., mortality, growth as dry weight, fecundity), as well sex ratios of offspring to detect reproductive impairment (Taylor et al 1999; Sanchez and Tarazona 2002; Depledge and Billinghurst 1999; LeBlanc et al. 1999; Hutchinson et al. 1999; Ingersoll et al. 1999). Survival and the total number of neonates per female were observed and recorded daily. Third and sixth brood neonates from the studied generations were counted, mass cultured by brood for 5 days, and then fixed in 95% ethanol followed by storage in a 70% ethanol solution (Black and Dodson 2003). The preserved neonates were subsequently examined for determination of sex ratios and dry weight.

Biochemical Response Variables

Vitellin

The upregulation of the egg yolk protein, vitellogenin, has been widely been used as a biomarker of estrogenic exposure in fish over the past 10 years (Volz and Chandler 2004). Vitellin (Vt), the egg yolk protein in invertebrates, could also provide a marker of hormone function (Oberdörster et al. 2000b) and endocrine disruption (Goto and Hiromi 2003) in daphnids as multiple vitellin-related endocrine cascades are potentially responsive to toxicant exposure (Volz and Chandler 2004). However, standardized quantitative assays for vitellin in invertebrates are lacking. Vitellin was measured in this study in both generations after approximately 21 days of exposure to detect biochemical

modulation of endogenous steroids. Organisms were collected immediately following release of a brood for Vt measures in an attempt to diminish potential variability among adult Daphnia. While several studies have investigated whether xenobiotics can abnormally induce vitellogenin in fish, very few have been performed with Crustacea (Volz and Chandler 2004).

Preliminary studies were conducted to determine the number of whole organisms needed to perform each assay. Vt from *D. magna* was purified and measured to serve as a positive control. Methods used were a modification of methods developed by Lui and O'Connor (1977), Lee and Walker (1995), and Oberdörster et al. (2000b). Gravid females, obtained from the culture at Baylor University, were collected immediately following release of their third brood. Excess water was removed and whole organisms were homogenized in 3X volume of extraction buffer (0.5 M NaCl, 5 mM EDTA, 0.5 M Tris-HCl, pH 7.0, 0.001% PMSF in isopropyl alcohol) with a tissue tearor for 15 seconds. The homogenate was centrifuged at 12,000 g for 10 minutes at 4^{o}C, the supernatant taken, and centrifuged again at 8,000 g for 30 minutes at 4^{o}C to make crude homogenates. The extracts were further separated on a Sephadex G-200 column with a flow rate of 15 mL minute^{-1} of phosphate buffer to isolate fairly pure Vt.

Western blots were performed using the partially purified Vt to measure the protein content of various numbers of *D. magna.* Proteins were separated on a 6% SDS gel, transferred to a polyvinylidene difluoride (PVDF) membrane and immunoblotted. Proteins were separated on a 6% SDS-polyacrylamide gel at 200 V for approximately one hour or until the dye reached the bottom of the gel. The gel was stained with Coomassie blue to confirm transfer efficiency. The membrane was blocked overnight at 4^{o}C (PBS +

3% BSA). After rinsing four times with PBST (PBS + 0.05% Tween 20) for 10 minutes each, the blot was incubated with the monoclonal antibody S-15-2 (Oberdörster et al. 2000b). The antibody was diluted 1:3 with PBST. Membranes were incubated for 2 hours at room temperature on a gentle rocker. The blot was then rinsed four times with PBST for 10 minutes each and incubated for 1 hour with a secondary antibody, anti-mouse antibody (1:5,000 dilution in PBST) at room temperature on a gentle rocker. The membrane was then rinsed four times in PBST for 10 minutes each and then incubated for 1 minute in Enhanced Chemiluminescence Reagent. Multiple exposures of Kodak X-OMAT film were performed and film was developed using a X-ray film processor. Based on these preliminary studies, it was determined that a minimum of two adult *D. magna* were needed to perform the Vt analyses.

Vt was measured in both generations of all multigeneration studies after approximately 21 days. Six gravid females were collected immediately following release of their seventh brood. Excess water was removed and whole organisms, pooled in groups of two, were homogenized in 300 µL extraction buffer (0.5 M NaCl, 5 mM EDTA, 0.5 M Tris-HCl, pH 7.0, 0.001% PMSF in isopropyl alcohol). The homogenate was centrifuged at 12,000 g for 10 minutes at 4^{o}C, the supernatant taken, and centrifuged again at 8,000 g for 30 minutes at 4^{o}C to make crude homogenates. These homogenates were then stored at -20^{o}C for analysis following the completion of all multigeneration experiments.

Slot-blots for Vt were done using the thawed crude homogenates. Methods used were a modification of methods developed by Oberdörster et al. (2000a). The PVDF membrane was wetted in methanol, rinsed in water for 5 minutes, and used in the BioRad

Bio-Dot® SF Microfiltration Apparatus. Two rinses of 100 μL PBS were done under mild vacuum, and samples were applied to each well. A standard curve using the partially purified Vt from daphnids made during preliminary studies (3.75-75 μg protein) was used to quantify Vt from samples. 100 μL of sample in duplicate were applied to each well after heating 150 μL of homogenate with 50 μL of sample buffer (0.8 M Tris-HCl, pH 6.8, 10% glycerol, 2% sodium dodecyl sulfate (SDS), 5% w/v bromophenol blue) and 10 μL of double-deionized water, obtained from a $MilliQT_{plus}$ Ultra-Pure Reagent Water System, at 95°C for 5 minutes. An additional 100 μL PBS was added to each well, samples were allowed to gravity filter through the apparatus for 2 hours at room temperature, and the membrane was blocked overnight in PBS plus 3% bovine serum albumin at 4°C. After rinsing 4 times with PBS plus 0.05% Tween 20 (PBST), the membrane was incubated for 2 hours at room temperature with a 1:2.5 dilution of S-15-2 monoclonal anti-Vt antibody (Oberdörster, Rice, and Irwin 2000), rinsed 4 additional times with PBST, and incubated 1 hour at room temperature with 1:10,000 dilution of antimouse IgG coupled to horseradish peroxidase secondary antibody. After the membrane was rinsed 4 times with PBST, slots were visualized using Enhanced Chemiluminescence Reagent. Multiple exposures of Kodak X-OMAT film were performed, film was developed using a X-ray film processor, and the developed film was analyzed using an Alphalmager 2200 spot densometer (Alpha Innotech Corporation, San Leandro, CA)

Ecdysone

Ecdysone, a molting hormone that is structurally similar to vertebrate estrogens (Segner et al. 2003), is critical to normal embryo development and the production of

viable offspring (Mu and LeBlanc 2002a). While assessment of this endogenous steroid is useful in detecting endocrine modulation by environmental contaminants, few studies have utilized ecdysone modulation as a biochemical response or biomarker to xenobiotic exposure in *D. magna* as standard methods do not yet exist (Mu and LeBlanc 2004; Mu and LeBlanc 2002a). Ecdysone was measured in this study in both generations after approximately 21 days of exposure.

Ecdysone was measured in the thawed crude homogenates using a 20HE Enzyme Immunoassay (EIA) kit (Cayman Chemical, Ann Arbor, MI). Preliminary studies were conducted to determine if an extraction step would be required to isolate ecdysone from the homogenate for use in the EIA kit. A trial EIA assay was conducted with 2 standard curves in duplicate, one made in EIA buffer required by the kit and one made in homogenization buffer. The results from this preliminary test showed that the EIA buffer and homogenization buffers matched and no extraction step would be necessary. Sample analysis was thus conducted using the same crude homogenate samples used in Vt analysis.

Ecdysone was measured generally according to the standard protocols provided with the 20HE EIA kit (Cayman Chemical 2002). This assay is based on the recognition of 20HE by specific monoclonal anti-rabbit antibodies. 20HE present in the sample and an acetylcholinesterase (AchE) conjugate (tracer) are premixed and added into each well of a microplate, allowing 20HE and the tracer to compete for a limited number of binding sites of the anti-rabbit antibodies immobilized on the surface of the wells. When the concentration of the 20HE is higher relative to the tracer, the 20HE will predominantly bind the antibody and vice versa. The plate was washed to remove any unbound reagents

and Ellman's Reagent, containing the substrate AchE, was added to the well to develop a distinct color that absorbs strongly at 412 nm. The intensity of the color, determined spectrophotometrically, was inversely proportional to the amount of 20HE present in the well during incubation (Cayman Chemical 2002).

Intrinsic Rate of Population Growth

This study also examined the intrinsic rate of population growth (*r*), an endpoint recommended in toxicological studies because it combines lethal and sublethal effects into one meaningful measure (Stark et al. 1997). *r* integrates the measures of age-specific survival and fecundity to estimate the effect of toxicant exposures on population growth. *r* is calculated using successive approximations of Lotka's formula (Lotka 1913):

2.1 $$\sum l_x m_x e^{-rx} = 1$$

where l_x is the proportion of individuals surviving to age x, m_x is the age-specific fecundity (mean number of neonates produced per surviving female at age x), and x is expressed in days. The exponent *r* was estimated in both generations by iteration until a value is found so that the calculated value of $l_x m_x e^{-rx}$ summed over 21 days was equal to 1. The value for *r* calculated in *D. magna* after 21 days is indistinguishable from *r* estimated for the entire life span, due to the importance of early reproduction (Van Leeuwen et al. 1985). Several researchers have advocated the use of *r* to estimate chronic toxic effect in *D. magna* at the population level (Van Leeuwen et al. 1985; Day and Kaushik 1987; Munzinger 1990; Ferrando et al 1993; Ferrando et al. 1995; Sanchez et al. 1999; Sanchez et al. 2000; Villarroel et al. 2000; Muyssen and Janssen 2001; Pane et al. 2004).

Statistical Analysis

EPA Probit Method, the Spearman-Karber Method, and the Trimmed Spearman-Karber Method, designed to produce LC50 values (the lethal concentration to 50% of test organisms) and associated 95% confidence intervals, were used to obtain estimations of LC50 values. TOXSTAT, designed specifically for statistical analysis of toxicological data (e.g. reproduction), was used to initially perform all statistical analyses on chronic response variables. Subsequently, SAS was employed for all non-reproductive response variables (dry weight, *r*, Vt, and Ecdysone) (Version 8, Cary, NC, USA). One-way ANOVA and Dunnett's tests were used to analyze the individual responses to treatment levels of each compound. Shapiro-Wilk's and Bartlett's test procedures were utilized to validate that the observations within treatments were normally distributed and that the variance of the observations was homogenous across all toxicant concentrations and the control. When ANOVA assumptions could not be met, the Kruskal Wallis and Dunn's tests were used to analyze the individual responses to treatment levels of the compounds. A t-test was performed for each compound and response variable combination to determine if the solvent carrier control was significantly different from the RHW control; none of the solvent carrier controls were significantly different from RHW control measures.

CHAPTER THREE

Results

Acute Toxicity Tests

Preliminary range-finding toxicity tests were performed to identify the range of treatment levels of the compounds that would affect survivorship before performing multigenerational testing. The mean LC50 values observed for each compound at 96 hours were as follows: EE2 was 2590.38 $\pm$ 87.13 μg L^{-1} (n=2), 20HE was 592.54 $\pm$ 25.15 μg L^{-1} (n=2), and F was 129.39 $\pm$ 27.24 μg L^{-1} (n=2). Survivorship was not affected at the T treatment levels tested, ranging from 125 to 2000 μg L^{-1}. Sublethal treatment levels for transgenerational studies were chosen based on these LC50 values.

Multigenerational Toxicity Studies

Reproduction

A significant (p<0.05) decrease in reproduction was observed in early broods of the F0 generation at all treatment levels of EE2 (Figure 3) and 20HE (Figure 4), and in the higher treatment levels of F (Figure 5) and T (Figure 6). With the exception of T, later broods of F0 organisms were not statistically different from the control. 20HE treatment levels that produced significant reductions in F0 brood size did not significantly reduce fecundity in the F1 generation. Treatment levels of EE2, F, and T that significantly reduced fecundity in F0 broods also significantly (p<0.05) reduced fecundity in the F1 generation, but the effect was observed generally in earlier broods and/or higher treatment levels than in the F0 (Figures 7-10).

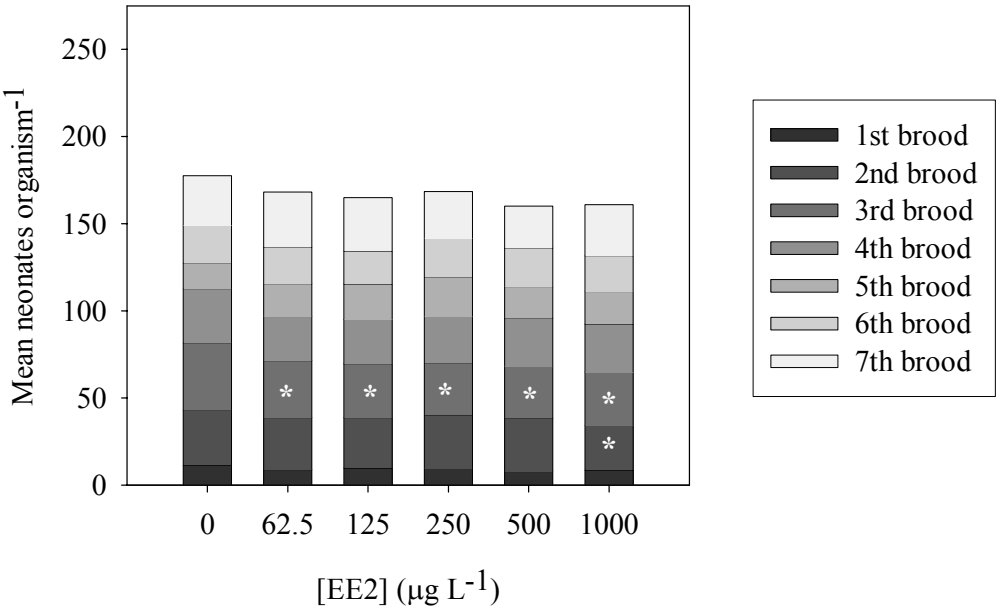

Fig. 3. Effects of EE2 on brood sizes in the F0 generation of *D. magna* (*$p \leq 0.05$; N=10).

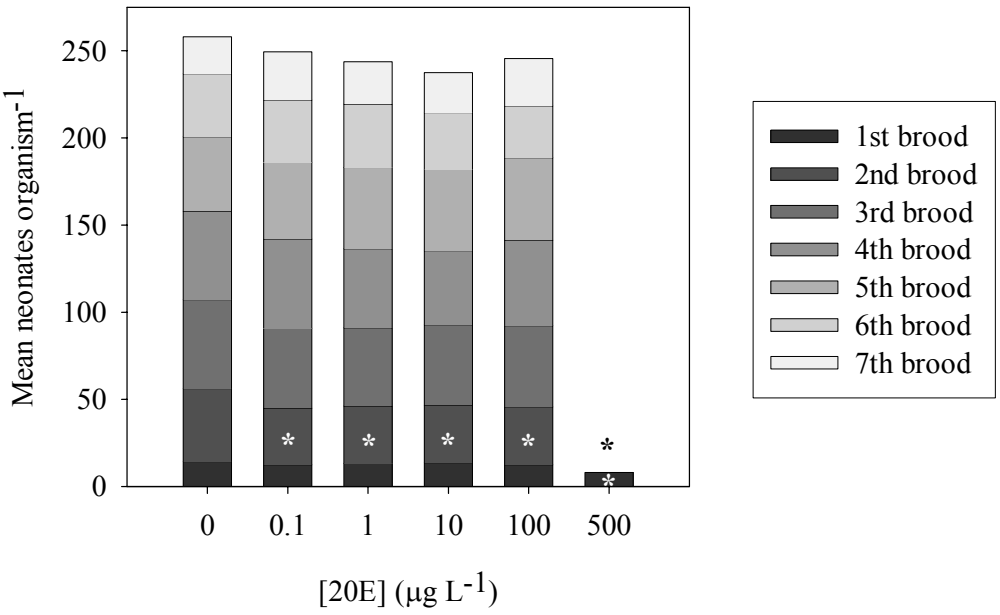

Fig. 4. Effects of 20HE on brood sizes in the F0 generation of *D. magna* (*$p \leq 0.05$; N=10).

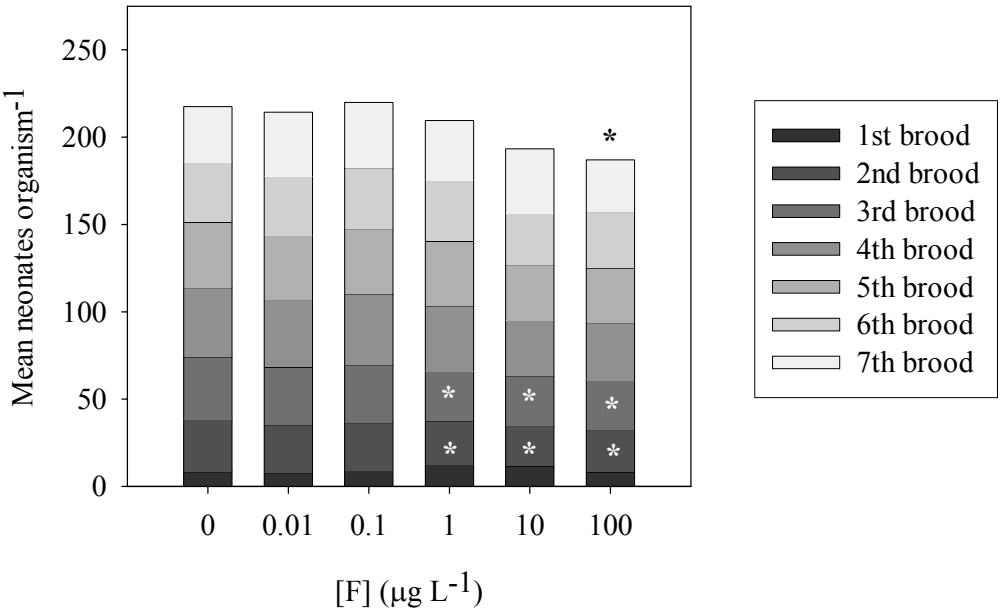

Fig. 5. Effects of F on brood sizes in the F0 generation of *D. magna* (*$p \leq 0.05$; N=10).

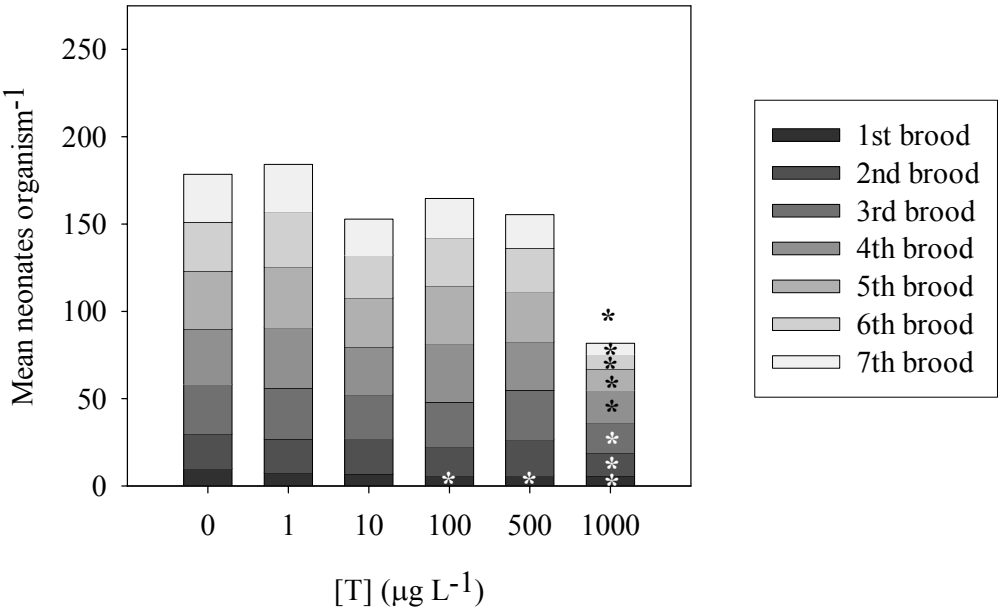

Fig. 6. Effects of T on brood sizes in the F0 generation of *D. magna* (*$p \leq 0.05$; N=10).

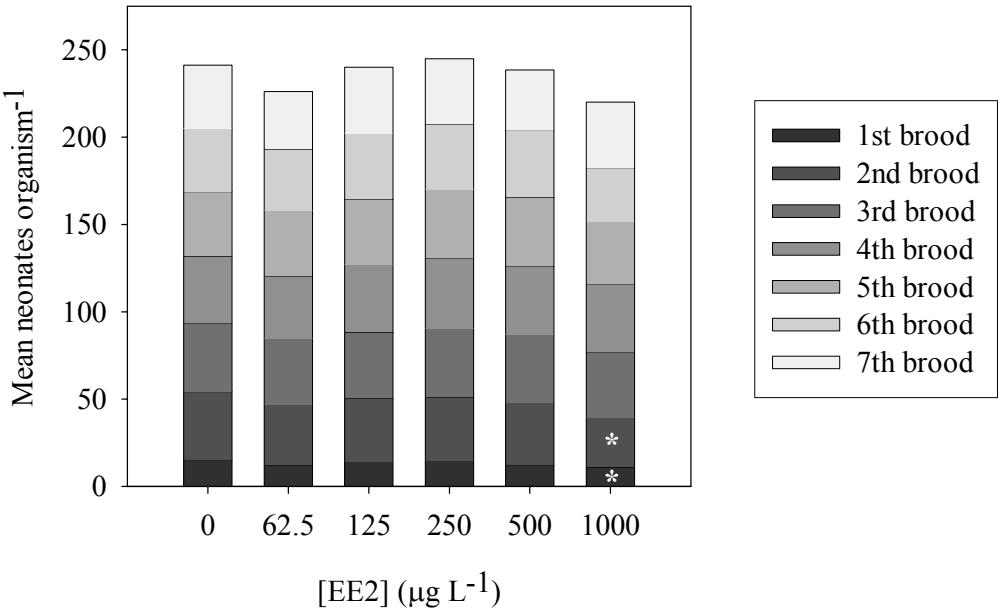

Fig. 7. Effects of EE2 on brood sizes in the F1 generation of *D. magna* (*$p \leq 0.05$; N=10).

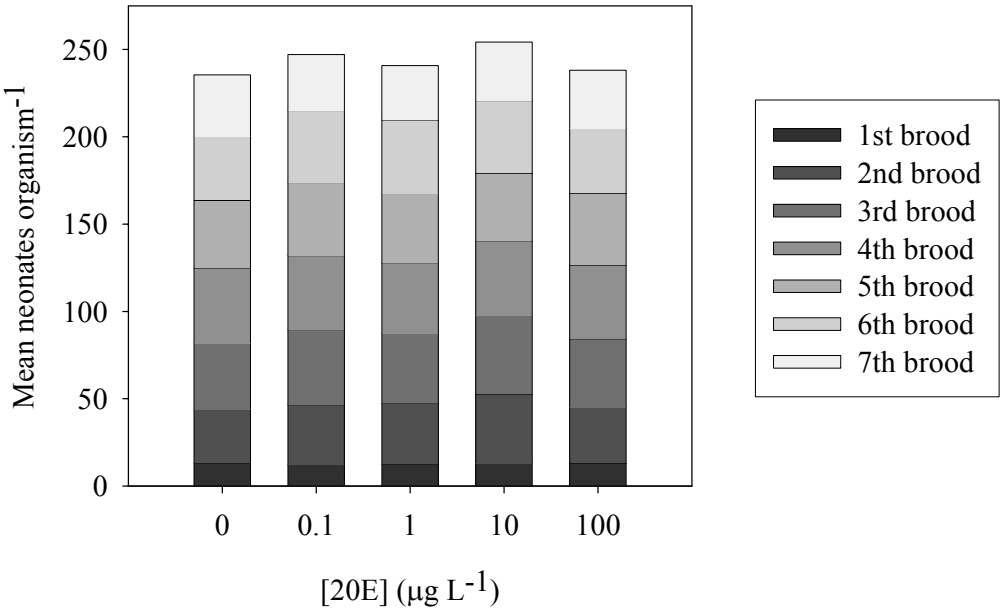

Fig. 8. Effects of 20HE on brood sizes in the F1 generation of *D. magna* (*$p \leq 0.05$; N=10).

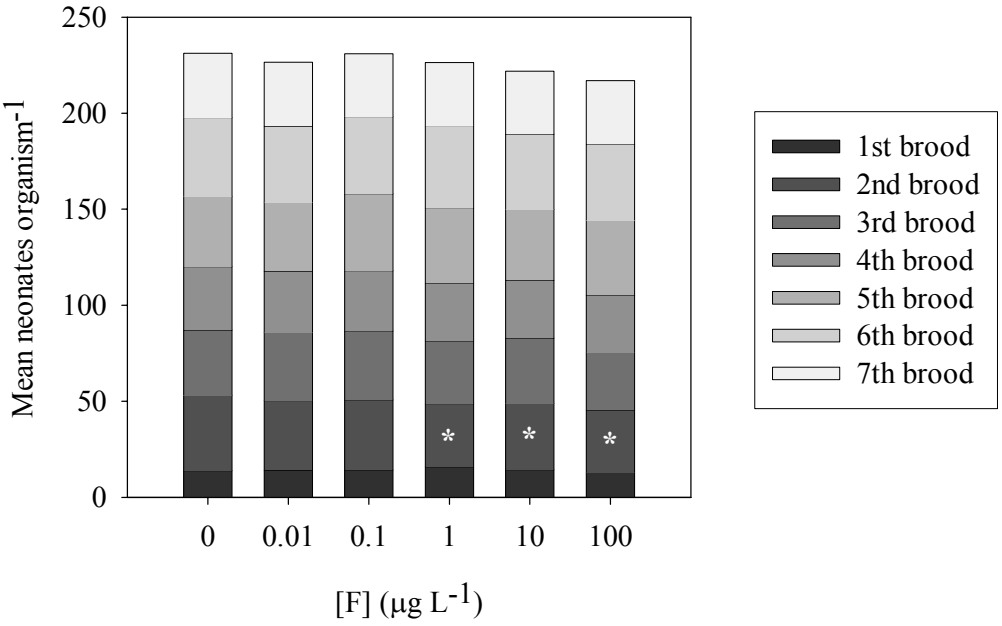

Fig. 9. Effects of F on brood sizes in the F1 generation of *D. magna* (*$p \leq 0.05$; N=10).

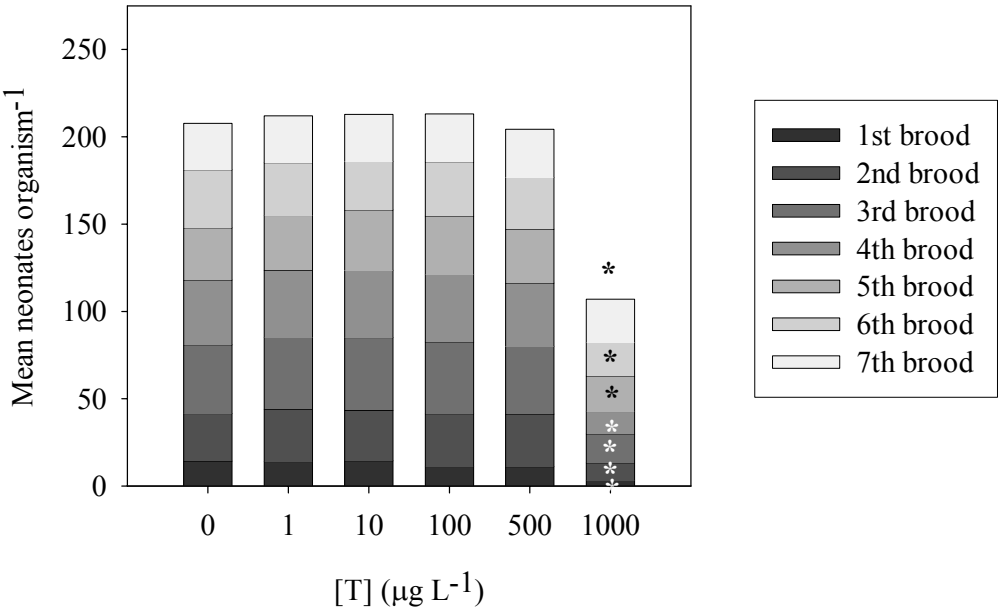

Fig. 10. Effects of T on brood sizes in the F1 generation of *D. magna* (*$p \leq 0.05$; N=10).

Overall fecundity (mean neonates female^{-1} over 21 days) was not significantly (p<0.05) affected in F0 of EE2 at any treatment level (Figure 4). 20HE, F, and T significantly (p<0.05) decreased 21-day F0 fecundity in the highest treatment levels only (Figures 5-7). Twenty-one day fecundity was not significantly (p<0.05) affected in F1by any treatment levels of EE2, 20HE, or F (Figures 8-10). T significantly (p<0.05) decreased overall fecundity in the highest treatment level of F1 (Figure 11).

Sex Ratio

Neonates from F0 and F1 organisms exposed to EE2 and 20HE were 100% female (Tables 2 and 3). A small percentage of males were observed in the higher treatment levels of F (>10 μg L^{-1}) and T (>100 μg L^{-1}) in F0 third (F < 2.5% male; T < 8.9% male) and sixth brood neonates (F < 2.2% male; T < 7.6% male) (Tables 4 and 5); the percentages of males generally decreased in the F1 at the same treatment levels (Tables 4 and 5).

Dry Weights

A statistically significant decrease in dry weights was observed in the third brood neonates of the F0 generation at all treatment levels of 20HE (p= 0.0002, Table 3) and T (p= 0.005, Table 5), however, this reduction was not observed in the F0 sixth brood neonates. A statistically significant decrease was observed in the third brood neonates of the F1 generation at higher treatment levels of 20HE than affected F0 neonates (p= 0.0019, Table 3), however, no significant decrease was observed in the F1 sixth brood neonates (p= 0.0817). No statistically significant decreases were observed in the neonates of the F1 generation exposed to T (third brood p= 0.7425; sixth brood p=

0.3941) or the neonates of the F0 (third brood p= 0.3019; sixth brood p= 0.2911) and F1 (third brood p=0.1545) generations exposed to F. Dry weights were unable to be collected from the neonates of organisms exposed to EE2.

Table 2. Responses of EE2 on F0 (top) and F1 (bottom) neonate production (± SD; *$p \leq 0.05$; N=10), sex ratio, and dry weight (± SD; *$p \leq 0.05$; N=3). NC = Not Collected.

EE2 (µg/l)	No. neonates per female	Brood no. significantly reduced	Sex ratio (% female neonates)		Dry weight (µg)	
			3rd brood	6th brood	3rd brood	6th brood
0.0	177.5 ± 22.0	---	100	100	NC	NC
62.5	168.1 ± 12.6	3	100	100	NC	NC
125	164.9 ± 24.9	3	100	100	NC	NC
250	158.6 ± 36.3	3	100	100	NC	NC
500	157.7 ± 27.9	3	100	100	NC	NC
1000	160.9 ± 20.7	2,3	100	100	NC	NC

EE2 (µg/l)	No. neonates per female	Brood no. significantly reduced	Sex ratio (% female neonates)		Dry weight (µg)	
			3rd brood	6th brood	3rd brood	6th brood
0.0	241.2 ± 16.8	---	100	100	NC	NC
62.5	226.1 ± 12.6	---	100	100	NC	NC
125	239.4 ± 17.8	---	100	100	NC	NC
250	244.9 ± 23.8	---	100	100	NC	NC
500	238.4 ± 21.0	---	100	100	NC	NC
1000	220.1 ± 18.3	1,2	100	100	NC	NC

Table 3. Responses of 20HE on F0 (top) and F1 (bottom) neonate production (± SD; *$p \leq 0.05$; N=10), sex ratio, and dry weight (± SD; *$p \leq 0.05$; N=3). DBC= Died Before Collected.

20HE (µg/l)	No. neonates per female	Brood no. significantly reduced	Sex ratio (% female neonates)		Dry weight (µg)	
			3rd brood	6th brood	3rd brood	6th brood
0.0	258.0 ± 28.9	---	100	100	37.8 ± 3.0	63.9 ± 6.9
0.1	249.4 ± 31.2	---	100	100	27.2 ± 3.3*	60.5 ± 14.0
1.0	243.7 ± 37.9	2	100	100	23.6 ± 3.2*	58.4 ± 13.9
10	238.7 ± 30.3	2	100	100	22 ± 2.2*	56.5 ± 6.8
100	237.4 ± 44.2	2	100	100	24 ± 1.2*	61.0 ± 9.4
500	0.8 ± 0.0*	1	DBC	DBC	DBC	DBC

20HE (µg/l)	No. neonates per female	Brood no. significantly reduced	Sex ratio (% female neonates)		Dry weight (µg)	
			3rd brood	6th brood	3rd brood	6th brood
0.0	235.5 ± 20.8	---	100	100	47.6 ± 5.1	42.7 ± 3.0
0.1	247.1 ± 27.0	---	100	100	40.2 ± 3.0	48.2 ± 4.8
1.0	240.7 ± 21.0	---	100	100	40.4 ± 5.0	47.5 ± 5.2
10	256.3 ± 25.1	---	100	100	30.3 ± 5.2*	40.1 ± 2.2
100	228.1 ± 30.3	---	100	100	30.1 ± 1.4*	41.5 ± 2.1
500	DBC	---	DBC	DBC	DBC	DBC

Table 4. Responses of F on F0 (top) and F1 (bottom) neonate production (± SD; *$p \leq 0.05$; N=10), sex ratio, and dry weight (± SD; *$p \leq 0.05$; N=3). NC= Not Collected.

F (µg/l)	No. neonates per female	Brood no. significantly reduced	Sex ratio (% female neonates)		Dry weight (µg)	
			3rd brood	6th brood	3rd brood	6th brood
0.0	217.5 ± 16.1	---	100	100	44 ± 3.0	48.7 ± 7.3
0.01	215.4 ± 22.8	---	100	100	45 ± 6.5	53.5 ± 9.5
0	219.9 ± 15.9	---	100	100	49 ± 15.7	58.5 ± 10.7
1	207.8 ± 12.6	2,3	100	100	56 ± 5.8	50.8 ± 7.9
10	196.0 ± 23.6	2,3	98.6	99.2	60 ± 15.4	64.1 ± 7.0
100	190.6 ± 21.1*	2,3	97.5	97.8	58 ± 3.9	69.9 ± 22.1

F (µg/l)	No. neonates per female	Brood no. significantly reduced	Sex ratio (% female neonates)		Dry weight (µg)	
			3rd brood	6th brood	3rd brood	6th brood
0.0	231.2 ± 14.6	---	100	NC	57 ± 5.8	NC
0.01	226.5 ± 19.1	---	100	NC	49 ± 2.2	NC
0	230.9 ± 11.3	---	100	NC	50 ± 3.9	NC
1	226.3 ± 36.5	2	100	NC	54 ± 2.7	NC
10	223.6 ± 14.2	2	100	NC	50 ± 2.2	NC
100	217.8 ± 17.3	2	100	NC	48 ± 6.4	NC

Table 5. Responses of T on F0 (top) and F1 (bottom) neonate production (± SD; *$p \leq 0.05$; N=10), sex ratio, and dry weight (± SD; *$p \leq 0.05$; N=3).

T (µg/l)	No. neonates per female	Brood no. significantly reduced	Sex ratio (% female neonates)		Dry weight (µg)	
			3rd brood	6th brood	3rd brood	6th brood
0.0	182.9 ± 31.7	---	100	100	76 ± 1.5	67.9 ± 13.0
1.0	184.1 ± 34.7	---	100	100	56 ± 7.1*	53.4 ± 11.0
10	152.8 ± 45.5	---	100	100	61 ± 0.6*	68.9 ± 13.0
100	168.8 ± 62.7	1	97.4	100	62 ± 8.4*	53.8 ± 9.2
500	155.3 ± 34.1	1	95.1	97	57 ± 4.7*	63.8 ± 9.2
1000	81.7 ± 24.9*	1-7	91.1	92.4	51 ± 12.9*	58.6 ± 5.7

T (µg/l)	No. neonates per female	Brood no. significantly reduced	Sex ratio (% female neonates)		Dry weight (□g)	
			3rd brood	6th brood	3rd brood	6th brood
0.0	207.7 ± 13.8	---	100	100	39 ± 4.5	42.8 ± 2.0
1.0	213.0 ± 14.7	---	100	100	32 ± 6.8	48.3 ± 2.7
10	212.8 ± 15.5	---	100	100	41 ± 6.1	45.3 ± 7.4
100	216.4 ± 15.2	---	100	100	37 ± 7.2	39.7 ± 7.4
500	204.3 ± 11.7	---	100	100	40 ± 8.7	48.0 ± 5.9
1000	107.0 ± 23.3*	1-6	98.6	98.9	34 ± 1.6	42.1 ± 5.8

Biochemical Biomarkers

Vitellin. A statistically significant increase in Vt was observed in the F0 generation in the highest treatment level of EE2 (p= 0.0009, Figure 11) and a significant decrease was observed in the higher treatment levels of T (p= 0.0059, Figure 14). These effects were not observed in F1 organisms. There were no statistically significant effects on Vt levels in the F0 generation exposed to 20HE (p= 0.0832, Figure 12) and F (p= 0.3072, Figure 13). However, a statistically significant increase in Vt was observed in the F1 generation in the lower treatment levels of F (p= 0.0326, Figure 13).

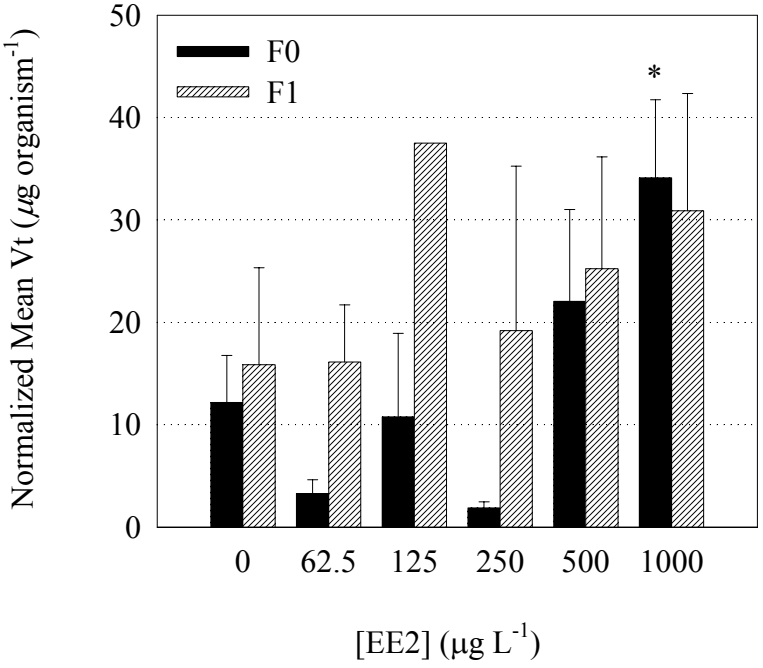

Fig.11. Effects of EE2 on the Vt level in the F0 and F1 generations of *D. magna*. (± SD; *$p \leq 0.05$; N=3).

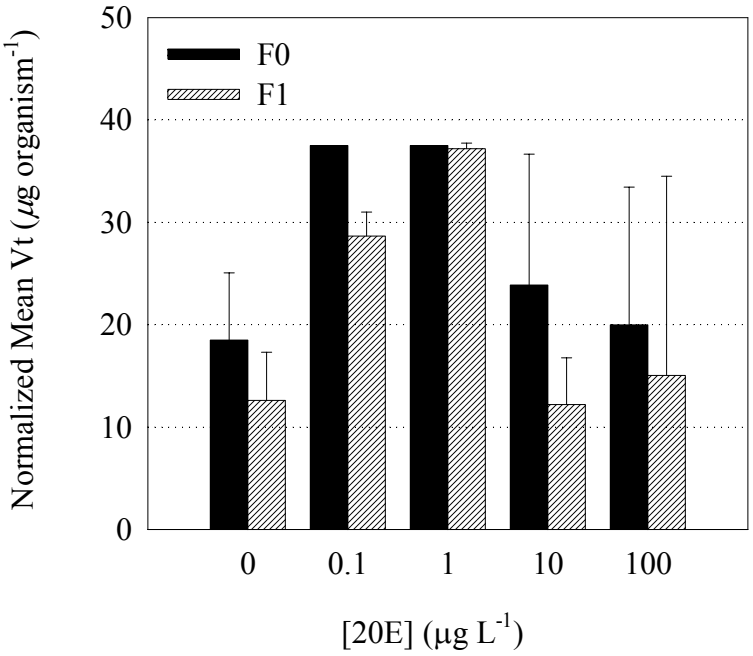

Fig.12. Effects of 20HE on the Vt level in the F0 and F1 generations of *D. magna*. (± SD; *$p \leq 0.05$; N=3).

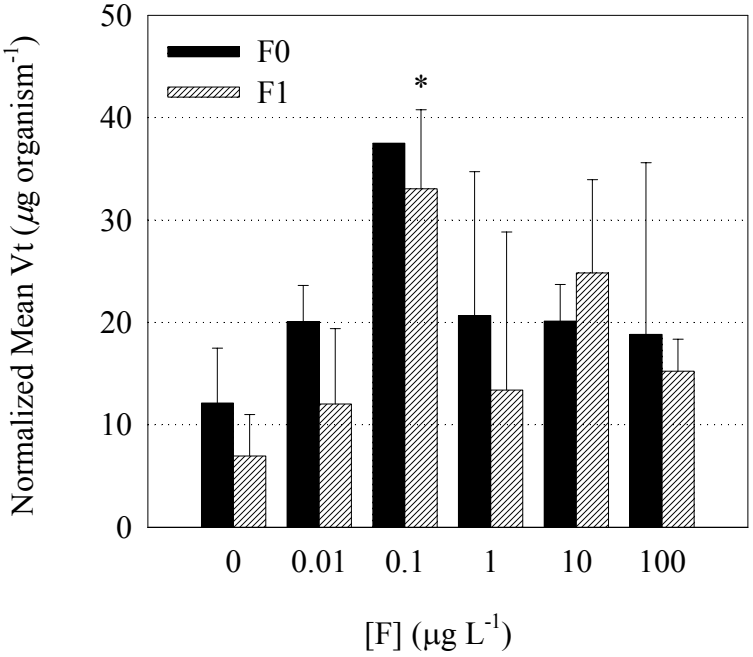

Fig.13. Effects of F on the Vt level in the F0 and F1 generations of *D. magna*. (± SD; *$p \leq 0.05$; N=3).

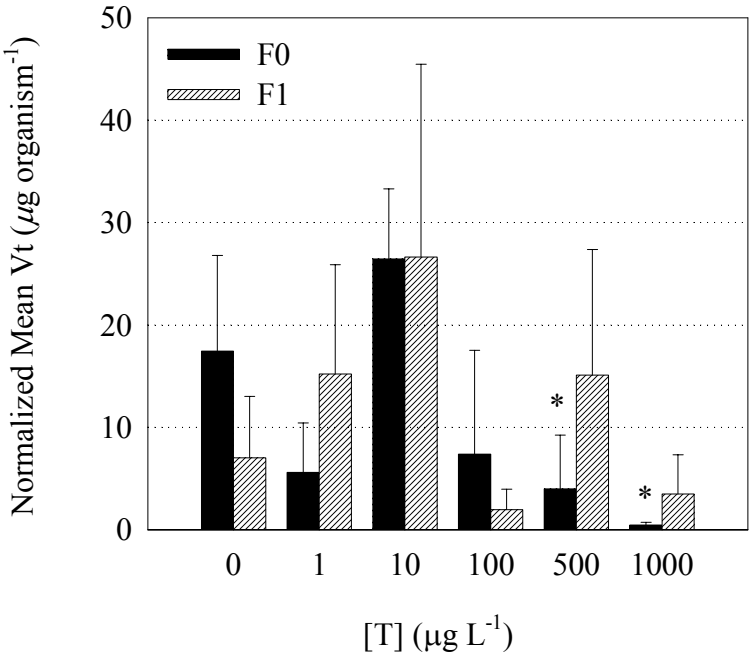

Fig.14. Effects of T on the Vt level in the F0 and F1 generations of *D. magna*. (± SD; *$p \leq 0.05$; N=3).

Ecdysone. A statistically significant increase in ecdysone was observed in the F0 and F1 generation in the highest treatment level of 20HE (F0 $p= 0.0111$; F1 $p= 0.0136$, Figure 15). A statistically significant decrease was observed in the F0 generation in the lowest treatment level of T ($p= 0.0339$, Figure 17); this reduction was not observed in the F1 generation. Ecdysone levels were not significantly affected at the EE2 (F0 $p= 0.4276$; F1 $p= 0.3450$, Figure 14) and F (F0 $p= 0.0696$; F1 $p= 0.2874$, Figure 16) treatment levels tested.

Intrinsic Rate of Population Growth

A statistically significant decrease in the *r* value was observed in the F0 generation in the highest treatment level of 20HE ($p< 0.0001$, Figure 20). This sharp reduction in population growth at the highest treatment level due to F0 mortality

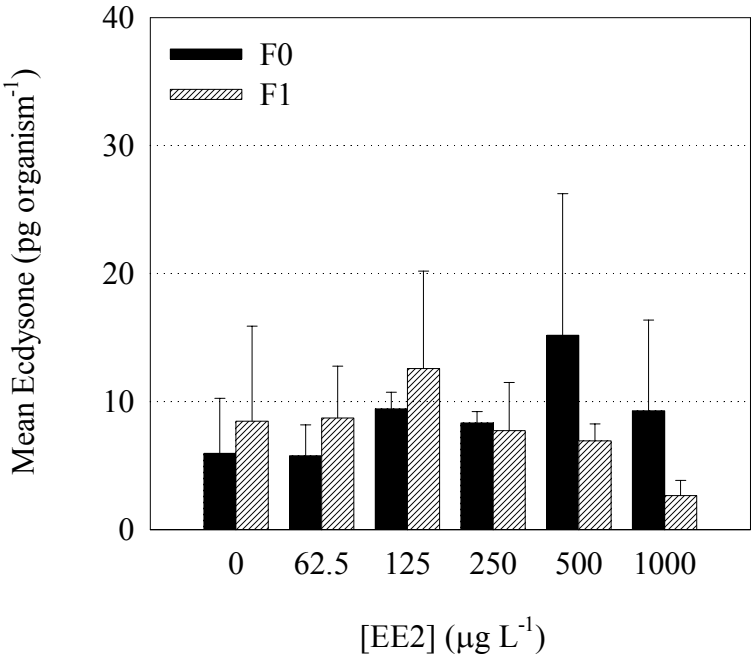

Fig.15. Effects of EE2 on the Ecdysone level in the F0 and F1 generations of *D. magna*. (± SD; *$p \leq 0.05$; N=3).

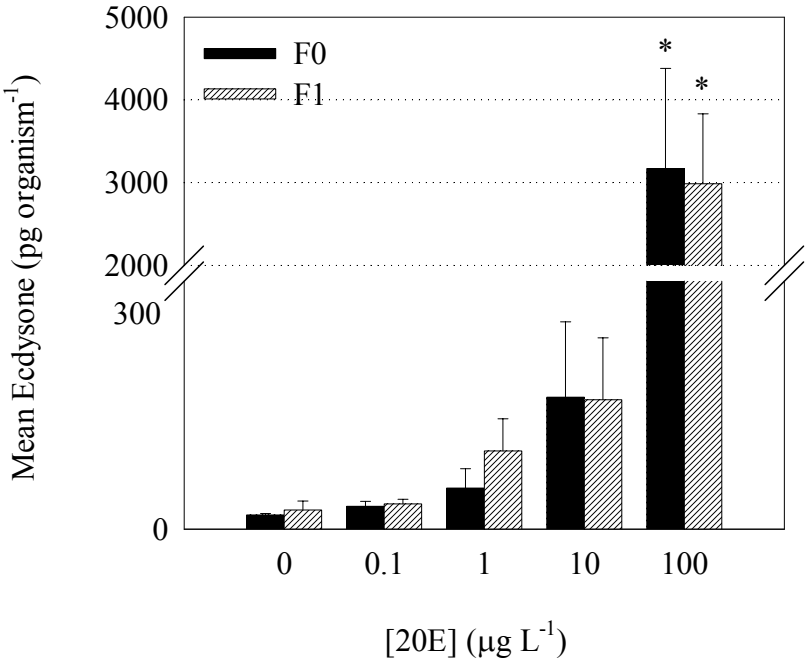

Fig.16. Effects of 20HE on the Ecdysone level in the F0 and F1 generations of *D. magna*. (± SD; *$p \leq 0.05$; N=3).

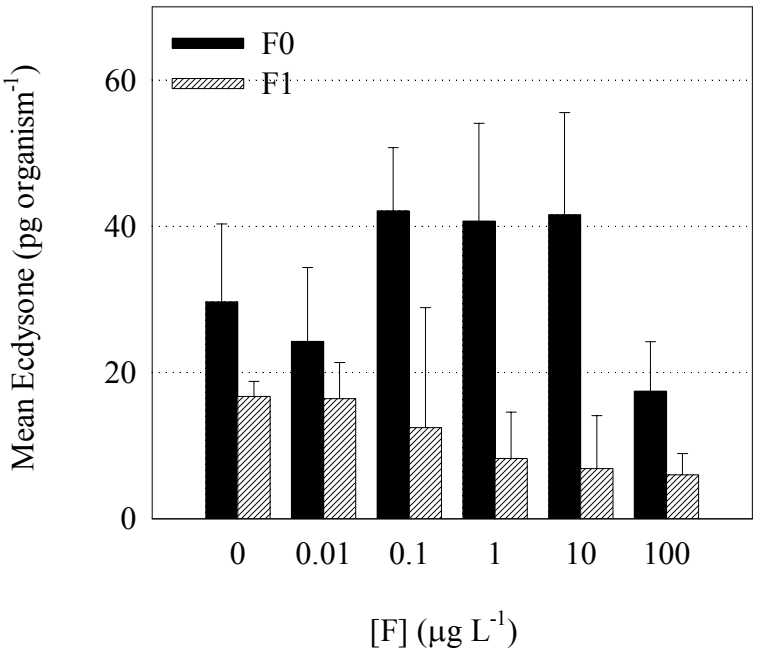

Fig.17. Effects of F on the Ecdysone level in the F0 and F1 generations of *D. magna*. (± SD; *$p \leq 0.05$; N=3).

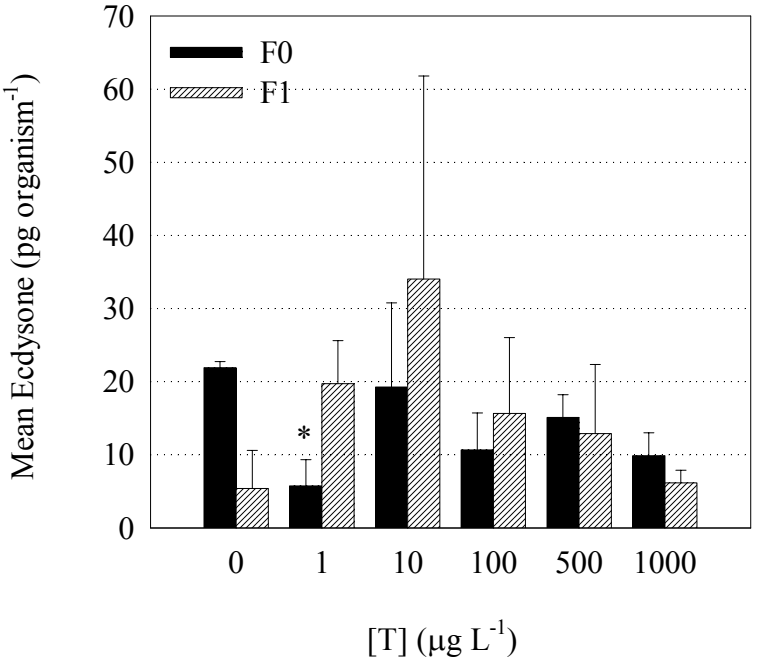

Fig.18. Effects of T on the Ecdysone level in the F0 and F1 generations of *D. magna*. (± SD; *$p \leq 0.05$; N=3).

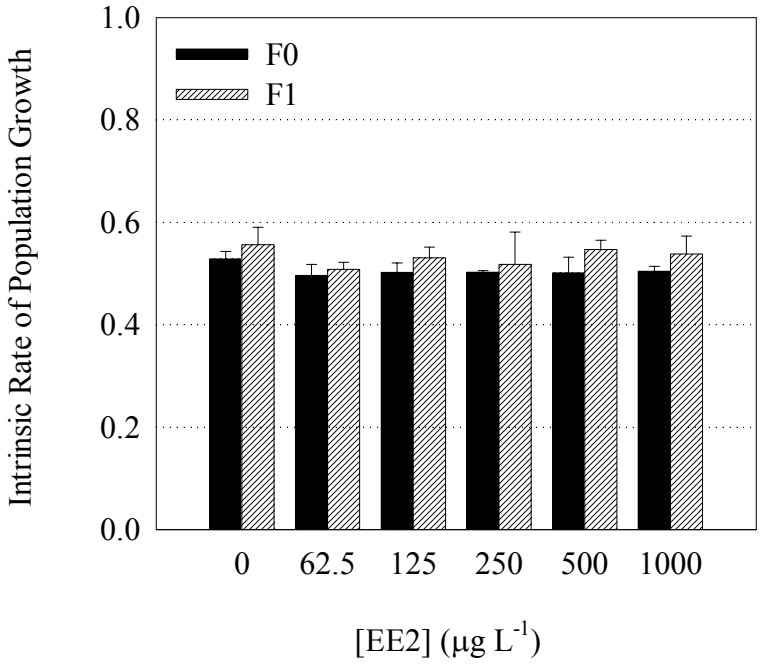

Fig. 19. Effects of EE2 on the intrinsic rate of population growth in the F0 and F1 generations of *D. magna*. (± SD; *$p \leq 0.05$; N=3).

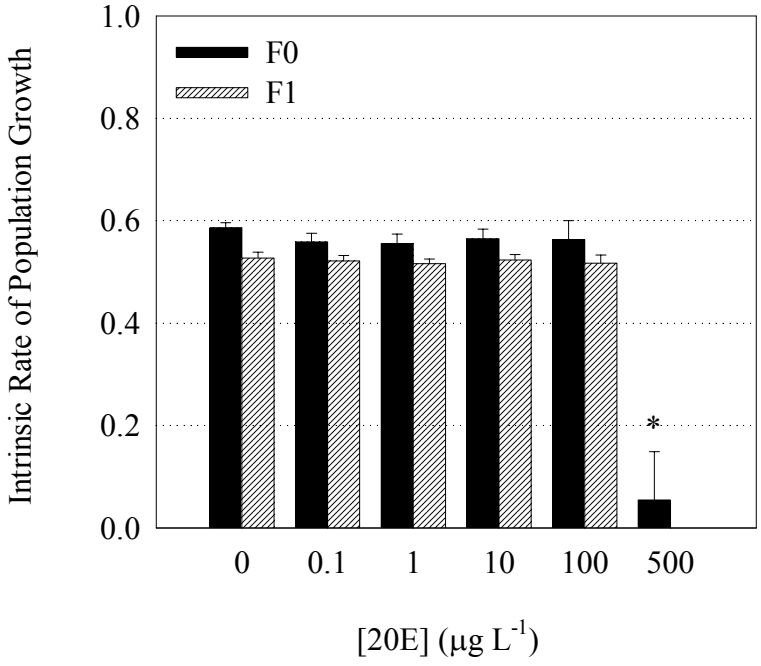

Fig. 20. Effects of 20HE on the intrinsic rate of population growth in the F0 and F1 generations of *D. magna*. (± SD; *$p \leq 0.05$; N=3).

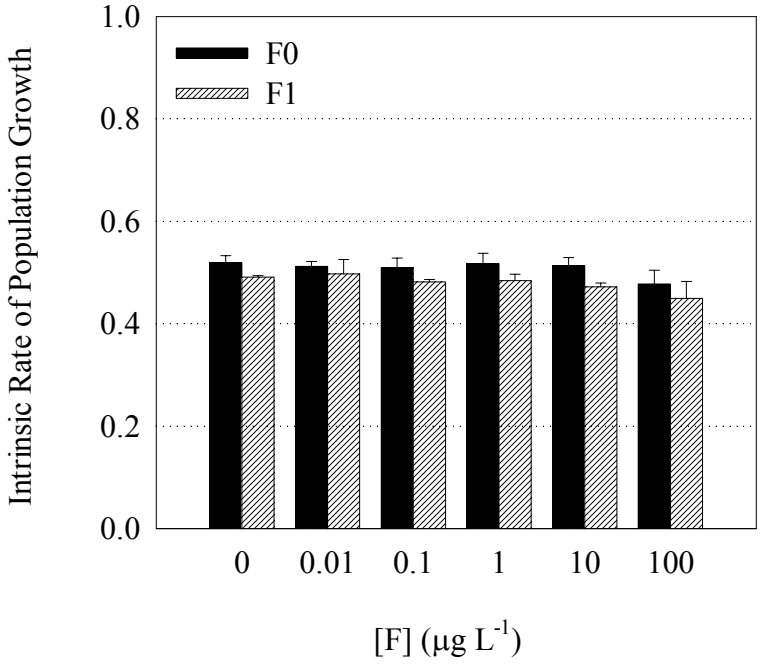

Fig. 21. Effects of F on the intrinsic rate of population growth in the F0 and F1 generations of *D. magna*. (± SD; *$p \leq 0.05$; N=3).

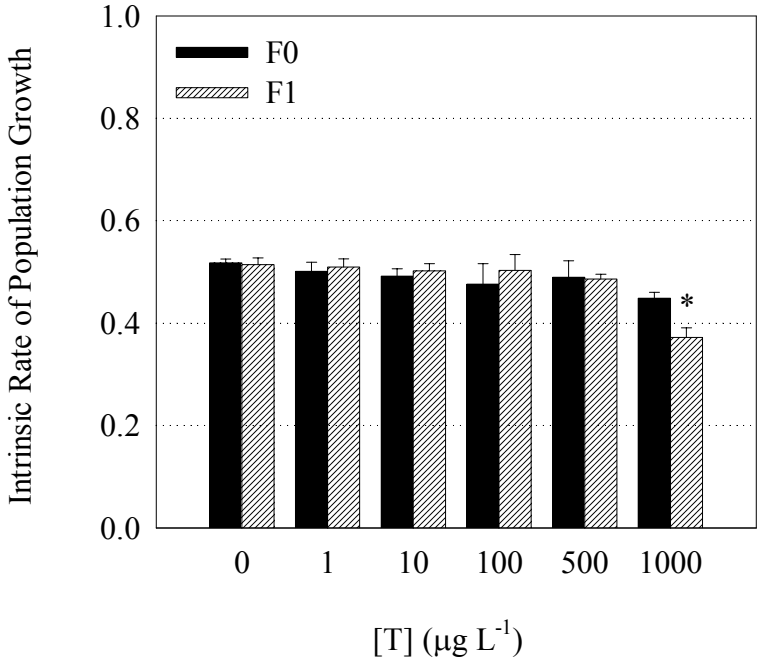

Fig. 22. Effects of T on the intrinsic rate of population growth in the F0 and F1 generations of *D. magna*. (± SD; *$p \leq 0.05$; N=3).

prevented the observation of F1 organisms exposed to 20HE. A statistically significant decrease was observed in the F1 generation in the highest treatment level of T ($p<$ 0.0001, Figure 23); this reduction was not observed in the F0 generation. Intrinsic rate of population growth values were not significantly affected at the EE2 (F0 $p=$ 0.3951; F1 $p=$ 0.5753, Figure 20) and F (F0 $p=$ 0.1257; F1 $p=$ 0.0960, Figure 22) treatment levels tested.

Routine Water Chemistry

All measured water chemistry parameters for each toxicity test fell within acceptable ranges for reconstituted hard water. pH ranged from 7.41 to 8.35. Dissolved oxygen ranged from 6.22 to 8.28 mg L^{-1}. Temperature ranged from 24 to 26^{o}C. Hardness ranged from 168 to 180 mg L^{-1} $CaCO_3$. Alkalinity ranged from 105 to 124 mg L^{-1} $CaCO_3$.

CHAPTER FOUR

Discussion

The primary objective of this study was to investigate the chronic effects of a model mammalian estrogen receptor agonist (EE2) and antagonist (F) on the transgenerational life-history of the model invertebrate *Daphnia magna.* A secondary objective of this study was to investigate *D. magna* biochemical biomarker responses to chronic EE2 and F exposure. Routine aquatic toxicity tests based on acute and/or chronic exposures form the basis of traditional ecological risk assessments, but these techniques alone may not be appropriate for potentially endocrine active compounds. Therefore, a multigenerational experimental design was employed in this investigation to account for continuous exposure to these compounds during all life history, to assess the long-term chronic effects over two generations, and to evaluate ecologically relevant population effects in a nontarget aquatic organism.

Life History and Population Responses

This study indicates short-term exposure (6-10 days) of EE2 and 20HE at all treatment levels and F and T at >1 μg L^{-1} and >100 μg L^{-1} respectively, reduced *D. magna* fecundity in the first generation, while longer-term exposures (>10 days) did not. In each exposure scenario, generally the earlier (1^{st}-3^{rd}) broods were significantly reduced in the number of females per brood while later broods in the same generation (F0) were not significantly different from the control. The only exception to this was at the highest treatment level of T, 1000 μg L^{-1},, in which all broods were significantly reduced. This

pattern of significantly affected early broods was also observed in dry weights of organisms exposed to 20HE and T and in sex ratios of F and T. Despite the significant reduction in early brood size across several treatment levels, the overall fecundity (neonates organism^{-1}) over 21 days was only reduced in the highest treatment levels of 20HE, T, and F.

A similar response was recently published in which adult *D. magna* exposed to 100 μg L^{-1} T for six days had an average of 32% fewer offspring than their reproductive controls, while this T-associated decline was not observed in long-term assays (Kashian and Dodson 2004). It has been demonstrated that T conjugation in invertebrates can serve as targets for endocrine disrupting chemicals (Janer et al. 2005) and that *Daphnia* are capable of hydroxylating T at multiple sites by P-450 enzymes (Baldwin and LeBlanc 1994). Cytochrome P-450s (CYP) are one of the major phase I-type classes of detoxification enzymes found in terrestrial and aquatic organisms ranging from bacteria to vertebrates. These enzymes metabolize a wide variety of substrates including endogenous molecules (fatty acids, steroids) and xenobiotics (hydrocarbons, pesticides, drugs) (Synder 2000). While several studies indicate P-450 activity in crustaceans, including daphnids, few crustacean CYPs have been characterized (David et al. 2003). Studies by Baldwin and LeBlanc (1994) suggest that daphnids express at least five distinct P450 enzymes involved in oxidative metabolism and indicate phase I and II biotransformation activity in daphnids. The majority of crustacean CYPs that have been isolated belong to the ubiquitous CYP4 family and their function has not been completely elucidated (David et al. 2003).

CYP-mediated metabolism may lead to xenobiotic detoxification resulting in acclimation. Therefore, acute exposure would have a greater affect on fecundity than a long-term, chronic exposure, as observed in this investigation. Kashian and Dodson (2004) hypothesized that chronic exposure may allow adequate time for CYP induction and upregulation prior to the release of the first brood. Based on observations from this investigation, it is more likely that the timing of CYP induction and upregulation is not prior to the first brood, but sufficiently early enough in the adult stage of *Daphnia* that no significant reductions are seen over a full chronic assay. Another study by Kashian (2004) indicated CYP associated acclimation in *Daphnia* occurring 7-12 days (the time period in which the second-third broods are released) following the initial exposure to the estrogenic pesticide toxaphene. This acclimation pattern was also observed in the present study in the second generation of *D. magna* in sex ratios, dry weights, and fecundity.

Transgenerational responses are observed in first and second generations exposed to the same compound. In general, the lower treatment levels at which reduced brood sizes were observed in the first generation are not significantly reduced in the second generation. In addition, less broods were significantly reduced in the second generation than in the first generation at the same treatment levels. For example, in the first generation daphnids exposed to 1000 μg L^{-1}T, broods 1-7 were significantly reduced. In the second generation exposed to 1000 μg L^{-1}T, broods 1-6 were significantly reduced. These transgenerational patterns were also observed in somatic growth and sex ratio measures.

Several multigenerational studies indicated that *D. magna* may develop resistance to heavy metals (Van Leeuwen et al. 1985; Munzinger 1990; Bodar et al. 1990;

Muyssen and Janssen 2001; Pane et al. 2004). In review of these studies, there are two main reasons why organisms exhibit resistance to pollutants: (1) They may have acquired tolerance by acclimatization during exposure to sublethal concentrations of that toxicant, employing metallothioneins in heavy metal exposure or cytochrome P-450 enzymes with other xenobiotic exposure, for example; (2) Populations may have evolved a genetically based resistance (Bodar et al. 1990). The sublethal responses of the second generation to exposure of all four compounds were lesser in degree and observed at higher treatment levels than in the first generation. While this observation suggests that a pattern of resistance, not solely the result of a physiological adaptation of each generation, no genetic measures where taken to confirm this observation. Another potential explanation is the first generation experiment started with neonates from previously unexposed parents, neglecting exposure during oogenesis and early embryogenesis. It has been demonstrated that the response of daphnids exposed to a toxicant from the earliest stages of development gives a better estimate of chronic toxicity than the response of the offspring of previously unexposed parents (Van Leeuwen et al. 1985). Studies have also found that the ability of neonatal organisms to metabolize xenobiotics can be elevated when organisms are exposed to chemicals prenatally (Baldwin et al. 1995).

In recent years there has been an increase in public awareness and demand to identify endocrine activity in a wide range of anthropogenic compounds and assess their effects on ecological receptors. Any chemical that affects an organism's fitness (survival, development, fecundity, or sexual determination) is likely to have effects that transcend individual responses and affect the entire ecosystem (Kashian and Dodson 2004). In this investigation, the intrinsic rate of population growth (r) was modeled to estimate

potential impacts at the population level of biological organization, which is becoming a critical component of population ecological risk assessments including cladocerans (Tanaka 2003). In this study, *r* was significantly decreased only at the highest treatment levels of 20HE (500 μg L^{-1}) in the first generation and T (1000 μg L^{-1}) in the second generation. Population level effects on *D. magna* were not observed following transgenerational exposures up to 1000 μg L^{-1} EE2 and 100 μg L^{-1} F. While this study has demonstrated that compounds designed to act on vertebrate estrogen receptors affect reproduction, growth, and sex determination in *D. magna*, the compounds tested in this study do not disrupt *D. magna* fitness at concentrations reflective of concentrations occurring in nature. However, other anthropogenic compounds may mimic hormones and potentially disrupt normal physiology and impact natural daphnids populations at varying concentrations (Kashian and Dodson 2004).

Biochemical Response Variables

The secondary objective of this study was to investigate the chronic effects of a mammalian estrogen receptor agonist and antagonist on biochemical biomarkers in *Daphnia magna.* While this investigation was not designed to identify mechanisms of action of EE2 or F in invertebrates, potential indictors for the mechanisms of action of EE2 and F were noted.

Ecdysteroids are invertebrate steroid hormones involved in the initiation of molting and egg maturation in many invertebrates (LeBlanc et al. 1999). If compounds designed to act on the vertebrate estrogen receptor (EE2 and F) were acting through the invertebrate ecdysone receptor, it is expected that comparative patterns in the modulation of Vt and ecdysone levels would exist between agonists (EE2 and 20HE) and antagonists

(F and T). For example, ecdysone receptor agonistics would cause effects attributed to hyperecdysonism (constant 20HE), such as increased levels of ecdysone and Vt (LeBlanc et al. 1999). Effects of ecdysone receptor antagonistics would mimic those observed when 20HE levels are low or absent, such as decreased levels of ecdysone and Vt (LeBlanc et al. 1999). Only a few studies have investigated the effect of xenobiotics on Vt or ecdysone levels in small invertebrates, such as *D. magna* (Volz and Chandler 2004; Mu and LeBlanc 2004; Mu and LeBlanc 2002a); thus, the mechanisms by which vertebrate estrogen receptor agents exert toxicity on invertebrates remain elusive.

Results regarding the effects of EE2 and F on Vt and ecdysone modulation compared to 20HE and T in *D. magna* were characterized by variable, non-monotonic biochemical responses. While a significant increase in Vt was observed in the first generation at the highest treatment level of the estrogen receptor agonist EE2, there were no significant Vt affects with exposure to 20HE. Additionally, while one mid-range treatment level of the estrogen receptor antagonist F indicated a statistically significant increase in Vt, the two highest treatment levels of the ecdysone receptor antagonist T resulted in significant decrease in Vt levels in the first generation. With regard to ecdysone levels, a significant increase was observed in the highest treatment level of 20HE in both generations and a significant decrease in the lowest treatment level of testosterone in the first generation.

Whereas the observed effects of EE2 and F on the biochemical biomarkers in this study do not conclusively suggest a target specific response, the use of acute:chronic ratios (ACRs) can provide some direction. An ACR is defined by 40 CFR §132.2 as a standard measure of the acute toxicity of a material divided by an appropriate measure of

the chronic toxicity of the same material under comparable conditions. A large ACR would thus represent a compound that exhibits impacts at much lower concentrations in chronic exposures with sublethal endpoints than in short-term lethality assays. For chemicals with a non-specific mode of action (e.g., narcosis), ACR values on the order of ≤ 10 are typical, while even for chemicals with more specific modes of action ACR values > 40 are rare (Rand 1995; Ankley et al. 2005). However, due to their low acute toxicity and relative specificity in terms of mode of action, certain groups of chemicals, such as those that activate the estrogen receptor in fish, have ACRs that are orders of magnitude higher than in traditionally tested aquatic contaminants (Ankley et al. 2005). The ACR values for each assay in this study are presented in Table 6. ACRs were calculated by dividing the LC50 (the lethal concentration to 50% of test organisms) by the NOEC (the concentration at which there was no observable effect at any endpoint in that assay).

Only those endpoints that accounted for the entire 21 day exposure period (overall fecundity, Vt levels, ecdysone levels, and intrinsic rate of population growth) were considered when calculating ACRs. Calculated ACRs were relatively small, ranging from approximately 1-60, indicating a less specific mode of action such as narcosis for EE2, F and T, but not 20HE.

The modulation of vitellin and ecdysone levels as might be expected in compounds acting through the ecdysone receptor were not observed with the compounds tested. There are potential reasons why these results did not indicate a clearer conclusion. Both Vt and ecdysone levels were measured in organisms collected after approximately 21 days. The experiment was intentionally designed in this manner to allow for

Table 6. Calculated acute:chronic ratios for each compound and generation in *D. magna*. The chronic response variable employed to calculate each specific ACR is designated by: [a] Overall fecundity; [b] Vitellin 2; [c] Ecdysone; [d] Intrinsic rate of population growth

Compound, Generation	96 hr LC50 ($\mu g\ L^{-1}$)	Chronic NOEC ($\mu g\ L^{-1}$)	Chronic LOEC ($\mu g\ L^{-1}$)	ACR (LC50/NOEC)
EE2 F0	2590	500	1000	5.18[b]
EE2 F1	2590	1000	>1000	2.59[a,b,c,d]
20HE F0	592	10	100	59.20[c]
20HE F1	592	10	100	59.20[c]
F F0	129	10	100	12.90[a]
F F1	129	100	>100	1.29 [a,b,c,d]
T F0	>2000	100	500	>20[b]
T F1	>2000	500	1000	>4[a,d]

observation of the daphnids throughout their lifecycle while also taking measurements of biochemical responses. The potential flaw in this design may have been these measurements were taken when the experiment ended, after *D. magna* were acclimated to experimental treatments and sublethal responses were minimal, if present at all. In addition, there was a large amount of variability within samples, as only six organisms were collected from each treatment levels and pooled to form a sample size of three, due to limited resources and logistical feasibility. While the results from this study do not clearly indicate the mode of action of EE2 and F in *D. magna*, sublethal parameters were identified that are perturbed by compounds designed to act on vertebrate estrogen receptors and highlight biologically significant transgenerational effects.

Conclusions

Results from this study suggest that the pharmaceuticals EE2 and F, model therapeutics designed to interact with vertebrate estrogen receptors, did not act through

the ecdysone receptor in *D. magna*. Thus, toxicity EE2 and F exerted on *D. magna* in this study likely resulted from non-endocrine-mediated responses. Regardless of the cause of impairment, sublethal and transgenerational impacts to *D. magna* were apparent and development of tolerance and potential resistance to these compounds was observed. Through modification of the standard *D. magna* chronic toxicity test and the monitoring of sublethal, biochemical endpoints that are reflective of endocrine processes, this research advanced the understanding of invertebrate impacts due to endocrine active pharmaceuticals, and potentially other endocrine disruptors and modulators. Specifically, results from this study will reduce uncertainty associated with ecological risk assessments of mammalian estrogen receptor agonist and antagonist pharmaceuticals by focusing future investigations on non-endocrine mediated responses in cladocerans, and potentially other invertebrates.

Ankley et al. (2005) recently summarized the relevant state-of-the-science in testing in an effort to better understand which methods from the wide range available are best suited for use in the regulatory environmental risk assessment of pharmaceuticals. Ankley et al.'s (2005) recommendations resulted from a Society of Environmental Toxicology and Chemistry Pellston Workshop, which brought together recognized leaders in the study of pharmaceuticals in the environment. This study employed several of the recommended testing methods, and extended beyond the scope of Ankley et al.'s (2005) recommendations. These included choosing a representation of a major phylum of concern from the standpoint of aquatic ecosystem protection and at least possible sensitivity to the class of pharmaceuticals of interest, evaluating a wide range of appropriate endpoints (biochemical to population level), and attempting to establish

whether the mode of invertebrate toxicity is related to the pharmacological mode of action in mammals and lower vertebrates (Ankley et al. 2005).

Ankley et al. (2005) also noted that although there are some ecotoxicity data for pharmaceuticals, the information is comprised largely of data concerning lethality in short-term assays across a comparatively broad array of chemical classes with various mechanisms of action. Because of the potential for continuous exposure of many compounds at low levels in nontarget aquatic organisms, particularly in effluent-dominated streams (Brooks et al. 2003), it is more relevant to perform chronic, life-cycle type tests that allow for assessment of long-term reproductive effects and encompass sensitive stages of organism development, subsequently decreasing hazard uncertainties in ecological risk characterization (Segner et al. 2003).

Recommendations

Based on the results of this investigation and the current state-of-the-science, several recommendations for future ecotoxicological testing and risk assessments concerning the effects of endocrine disrupting or modulating compounds and pharmaceuticals in invertebrates are presented below.

1. The results for short-term, long-term, and multigenerational test results in this study demonstrate that the expression of toxicity can vary with the duration of exposure. It is therefore crucial that the duration of the toxicity test accurately reflect real or potential environmental exposure. In order to establish realistic, protective environmental standards, appropriate time scales for toxicity tests must be identified that account for life cycle exposures.

2. The developmental stage at which the organism is exposed to a compound can potentially influence toxicological responses and the results of the toxicity test. *Daphnia,* like most organisms, may be particularly susceptible to toxicants during specific developmental periods, which may not fall within the time scale of an acute or chronic test (Colborn and Thayer 2000). The exposure duration of the toxicity test should also accurately reflect real or potential environmental exposure and account for the sensitive lifestages of test organisms.
3. A chronic reproduction test with a cladoceran (7 or 21 days depending on the species) has been recommended as a "base test" for assessing environmental risk of pharmaceuticals (Ankley et al. 2005). Additional multigeneration exposure testing should be considered when appropriate to help refine the risk assessment for a particular pharmaceutical. Multigeneration tests could be included based on several factors, including the physiochemical properties of the pharmaceutical, potential modes of action, and the exposure scenario. As demonstrated in this investigation, later generations exposed to a toxicant may give a better estimate of chronic toxicity than the response of the initially exposed organisms.
4. Use molecular and biochemical biomarkers in invertebrates as they become available. For example, an enzyme-linked immunosorbent assay (ELISA) was developed for lipovitellin quantification in copepods during this investigation that could be useful in future studies to reduce uncertainty in screening endocrine toxicity (Volz and Chandler 2004). To further delineate the potential for endocrine disruption in invertebrates, further efforts should attempt to evaluate *in vitro* data from hormone receptor binding assays (Dinan et al. 2001).

5. It has not been possible to conclude categorically that the biochemical effects observed in this study are receptor-mediated and result from an interaction with the endogenous endocrine system of invertebrates. Therefore, it is recommended that caution be exercised to avoid extrapolating results to other invertebrates in view of the limited knowledge of invertebrate endocrinology.

APPENDICES

APPENDIX A

1 Mix solutions Wash glassware	2 Start 1st gen test Load test Feed Water chemistry	3 Feed Wash glassware Record mortality Record neonates	4 Feed Renew solutions Record mortality Record neonates	5 Feed Wash glassware Record mortality Record neonates	6 Feed Renew solutions Record mortality Record neonates	7 Mix Solutions Feed Wash glassware Record mortality Record neonates
8 Feed Renew solutions Record mortality Record neonates Water chemistry	9 Feed Wash glassware Record mortality Record neonates	10 Feed Renew solutions Record mortality Record neonates	11 Feed Wash glassware Record mortality Record neonates	12 Start 2nd gen test Feed Renew solutions Record mortality Record neonates Preserve 3rd brood	13 Mix Solutions Feed Wash glassware Record mortality Record neonates	14 Feed Renew solutions Record mortality Record neonates Water chemistry
15 Feed Wash glassware Record mortality Record neonates	16 Feed Renew solutions Record mortality Record neonates	17 Feed Wash glassware Record mortality Record neonates	18 Feed Renew solutions Record mortality Record neonates	19 Mix Solutions Feed Wash glassware Record mortality Record neonates	20 Feed Renew solutions Record mortality Record neonates Water chemistry	21 Feed Wash glassware Record mortality Record neonates Preserve 6th brood
22 Feed Renew solutions Record mortality Record neonates Preserve 3rd brood	23 End 1st gen test Feed Wash glassware Record mortality Record neonates Preserve magna Homogenize magna	24 Feed Renew solutions Record mortality Record neonates	25 Mix Solutions Feed Wash glassware Record mortality Record neonates	26 Feed Renew solutions Record mortality Record neonates Water chemistry	27 Feed Wash glassware Record mortality Record neonates	28 Feed Renew solutions Record mortality Record neonates
29 Feed Wash glassware Record mortality Record neonates	30 Feed Renew solutions Record mortality Record neonates Preserve 6th brood	31 Mix Solutions Feed Wash glassware Record mortality Record neonates	32 Feed Renew solutions Record mortality Record neonates Water chemistry	33 End 2nd gen test Record mortality Record neonates Preserve magna Homogenize magna	34 Wash glassware	

Fig. A.1. Daily activities in multigenerational toxicity testing with *D. magna.*

APPENDIX B

Table B.1. Statistical tests and associated p-values for each non-reproductive response variable to EE2 F0 (top) and F1 (bottom). Enclosed values indicate $p \leq 0.05$; NC = Not Collected.

EE2 F0	ANOVA Assumptions Met	Statistical Test	*p*-value
Vitellin	Y	ANOVA, Dunnett's	0.0009
Ecdysone	Y	ANOVA, Dunnett's	0.4276
Dry weight, 3rd brood	NC	NC	NC
Dry weight, 6th brood	NC	NC	NC
Intrinsic Rate of Popln Growth	Y	ANOVA, Dunnett's	0.3951

EE2 F1	ANOVA Assumptions Met	Statistical Test	*p*-value
Vitellin	N	Kruskal Wallis, Dunn's	0.2365
Ecdysone	Y	ANOVA, Dunnett's	0.3450
Dry weight, 3rd brood	NC	NC	NC
Dry weight, 6th brood	NC	NC	NC
Intrinsic Rate of Popln Growth	Y	ANOVA, Dunnett's	0.5753

Table B.2. Statistical tests and associated p-values for each non-reproductive response variable to 20HE F0 (top) and F1 (bottom). Enclosed values indicate $p \leq 0.05$.

20HE F0	ANOVA Assumptions Met	Statistical Test	*p*-value
Vitellin	N	Kruskal Wallis, Dunn's	0.0832
Ecdysone	N	Kruskal Wallis, Dunn's	0.0111
Dry weight, 3rd brood	Y	ANOVA, Dunnett's	0.0002
Dry weight, 6th brood	Y	ANOVA, Dunnett's	0.5219
Intrinsic Rate of Popln Growth	Y	ANOVA, Dunnett's	<.0001

20HE F1	ANOVA Assumptions Met	Statistical Test	*p*-value
Vitellin	N	Kruskal Wallis, Dunn's	0.1037
Ecdysone	N	Kruskal Wallis, Dunn's	0.0136
Dry weight, 3rd brood	Y	ANOVA, Dunnett's	0.0019
Dry weight, 6th brood	Y	ANOVA, Dunnett's	0.0817
Intrinsic Rate of Popln Growth	Y	ANOVA, Dunnett's	0.7703

Table B.3. Statistical tests and associated p-values for each non-reproductive response variable to F F0 (top) and F1 (bottom). Enclosed values indicate $p \leq 0.05$; NC = Not Collected.

F F0	ANOVA Assumptions Met	Statistical Test	*p*-value
Vitellin	N	Kruskal Wallis, Dunn's	0.3072
Ecdysone	Y	ANOVA, Dunnett's	0.0696
Dry weight, 3rd brood	Y	ANOVA, Dunnett's	0.3019
Dry weight, 6th brood	Y	ANOVA, Dunnett's	0.2911
Intrinsic Rate of Popln Growth	Y	ANOVA, Dunnett's	0.1257

F F1	ANOVA Assumptions Met	Statistical Test	*p*-value
Vitellin	Y	ANOVA, Dunnett's	0.0326
Ecdysone	Y	ANOVA, Dunnett's	0.2874
Dry weight, 3rd brood	Y	ANOVA, Dunnett's	0.1545
Dry weight, 6th brood	NC	NC	NC
Intrinsic Rate of Popln Growth	Y	ANOVA, Dunnett's	0.0960

Table B.4. Statistical tests and associated p-values for each non-reproductive response variable to T F0 (top) and F1 (bottom). Enclosed values indicate $p \leq 0.05$.

T F0	ANOVA Assumptions Met	Statistical Test	*p*-value
Vitellin	Y	ANOVA, Dunnett's	0.0059
Ecdysone	Y	ANOVA, Dunnett's	0.0339
Dry weight, 3rd brood	Y	ANOVA, Dunnett's	0.0050
Dry weight, 6th brood	Y	ANOVA, Dunnett's	0.3552
Intrinsic Rate of Popln Growth	Y	ANOVA, Dunnett's	0.0580

T F1	ANOVA Assumptions Met	Statistical Test	*p*-value
Vitellin	Y	ANOVA, Dunnett's	0.1095
Ecdysone	Y	ANOVA, Dunnett's	0.1595
Dry weight, 3rd brood	Y	ANOVA, Dunnett's	0.7425
Dry weight, 6th brood	Y	ANOVA, Dunnett's	0.3941
Intrinsic Rate of Popln Growth	Y	ANOVA, Dunnett's	<.0001

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